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A Method for Determination of Low Carbon Monoxide Concentration in Blood

By

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Abstract

Most of the earlier described methods for the determination of carbon monoxide concentration in blood are not suited for the measurement of very low concentrations This is true particularly of spectrophotometric methods (Hartride 1922, Klendshoj [illegible]

Sjostrand 1951, Carlsten *et al* 1954) or the infrared CO meter (Lawther and Apthorp 1955, Gaensler *et al* 1957) Recently Coburn *et al* (1964) described an improved technique using the infrared CO meter, but this method too, has a comparatively large error in the range of normal CO content in blood (coefficient of variation about 6 per cent at a CO content of 0 1 ml CO/100 ml blood)

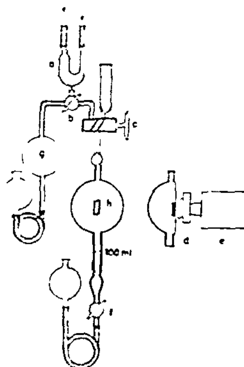


Fig. 1. The modified van Slyke apparatus. a) L tube with gas flowing to the CO meter. b), c) and f) stop-cocks. d) permanent magnet. e) electric motor. g) gas recipient. h) extraction chamber with teflon-coated magnet. For further explanation see text.

In this paper a method is described which allows the measurement of a low CO content of the blood using the technique for determination of CO in small gas samples with an improved hopcalite CO meter described by Linderholm and Sjöstrand (1956) and also a modified technique for extraction of CO from blood. In our hands this method seems to have a higher precision in the low range of blood CO content than earlier described methods. Various reagents and procedures for release of CO from the blood were tested.

Method

Apparatus for the extraction of CO from blood

A modified van Slyke apparatus was used for the extraction of CO from blood (Fig. 1). The extraction chamber had a volume of 100 ml. Stirring was performed with a small magnet coated with teflon, which was rotated by means of a magnet (d) outside the chamber and an electrical motor (e). Blood and reagents were admitted through the cup and the cock (c). By heightening the mercury leveling bulb, extracted gas could be ejected through the stopcocks to another gas recipient (g) with another mercury leveling bulb, and to the wide L tube (a) through which gas flows to the CO meter.

Reagents

1) Octylic alcohol. 2) 1 per cent saponin solution. 3) Different reagents used for release of CO from the hemoglobin, namely:

a) $K_3[Fe(CN)_6]$. 20 g was dissolved in distilled water to make 100 ml solution. A new solution was prepared every day. [illegible] ml of the $K_3[Fe(CN)_6]$ solution was used for up to 2 ml of blood and

period

For 1—2 ml of blood 10 ml, for 3—5 ml blood 15 ml and for 6—10 ml of blood 20 ml of the $KH(JO_3)_2$ solution were used. With this reagent the procedure for cleaning the van Slyke apparatus was easy. Some batches of $KH(JO_3)_2$ gave high blank values and had to be discarded.

Analytical procedure

The van Slyke apparatus (Fig. 1) was filled with mercury. A few drops of octylic alcohol were placed in the cup and drawn into the bore of the cock *c*. 5 ml of the saponin solution were added to the cup. Blood in an Ostwald pipette 0.3—10 ml of volume, was drawn under the saponin solution down into the extraction chamber, followed by 4 ml of the saponin solution. The Ostwald pipette was then immediately removed. The stopcock was closed and the rest of the saponin solution was sucked away. A reagent for the relaese of CO from the blood was added to the cup. By lowering the leveling bulb the mercury was drawn down to the lower part of the extraction chamber. Magnetic stirring was done during 1 min. By gentle opening of the stopcock *c* a defined amount of the CO releasing reagent in the cup was sucked down into the

In some analyses extraction and measurement of the released CO was done several times in succession. Ten minutes stirring periods were then used. When extractions were made in the presence of other gases (N_2, CO free air or O_2), about 20 ml of these were added through the cup.

The van Slyke pipette was cleaned with water, and now and then with a 30 per cent solution of KOH or NaOH followed by a 10 per cent HNO_3 solution and distilled water.

CO *Analysis*

Determination of the amount of CO in the gas extracted from the blood was done with the

van Slyke apparatus in as far as possible the same way as in the analysis of the blood samples, i.e. CO was added together with O_2, air, N_2, or in a very small gas volume.

The hopcalite CO meter is not quite specific to CO. Other gases which may be present in small amounts in blood such as acetone, ethanol and ammonia may influence the CO meter by oxidation or adsorption on the hopcalite. These factors may, however, be elliminated by using a water filter between the U tube of the van Slyke apparatus and the CO meter. The CO meter is less sensitive to methane than to CO.

Some of the results were checked by the method for analysis of CO in blood using palladium molybdenum test tubes (Linderholm 1965).

TABLE I. Comparison between different reagents for the release of CO from blood

Reagents compared	n	Blood sample: Volume ml	Blood sample: CO content range, ml CO/100 ml blood	$\bar{D}$ ml CO/100 ml blood
$KH(JO_3)_2$ – $K_3[Fe(CN)_6]$	8	1	0.07–0.34	0.005
$KH(JO_3)_2$ – $K_3[Fe(CN)_6]$	10	2	0.07–0.34	0.012[1]
$KH(JO_3)_2$ – $K_3[Fe(CN)_6]$	8	0.31	17.0–19.8	–0.21
$KH(JO_3)_2$ – H_2SO_4	8	1	0.07–0.34	0.005[1]
$KH(JO_3)_2$ – H_2SO_4	10	2	0.07–0.34	0.006[1]
$KH(JO_3)_2$ – H_2SO_4	6	0.31	17.0–19.8	0.18

$\bar{D}$ is the mean difference between the CO content of pairs of blood samples. Each of these samples was analyzed with one of two different reagents for comparison.

[1] $P < 0.05$

[2] $P < 0.01$

n is the number of blood sample pairs.

Results

Completeness of extraction of CO from blood

Blood samples of 0.3–2 ml containing 0.7–198 µl CO were analyzed using $K_3[Fe(CN)_6]$, H_2SO_4 and $KH(JO_3)_2$ to release CO from the hemoglobin. Extraction and stirring were done during successive 10-minute periods in "vacuum", i.e. with no other gas in the extraction chamber than that originating from the blood and reagent solutions. The amount of CO obtained in the second extraction period was 1.0–3.5 per cent (on an average about 2 per cent) of the total amount of CO extracted from the blood sample. After a third extraction no traces of CO were found. There was no significant difference between the different reagents in this respect.

Analysis of the gas collected from two extractions of 7 and 5 min (the standard procedure) resulted in the same amount of CO as the sum of the amounts of CO found after the first and second 10-min extraction periods.

The effect of the volume of the blood on the completeness of extraction of CO with the various reagents was examined by analyzing 1, 2 and 5 ml from one blood specimen. Identical results were obtained with 1 and 2 ml samples and also with 5 ml samples and $KH(JO_3)_2$. With 5 ml and $K_3[Fe(CN)_6]$ the CO concentration was only about 95 per cent and with H_2SO_4 only 76 per cent of the concentration expected from the analysis of 1 ml samples. After extraction of these larger blood samples with $K_3[Fe(CN)_6]$ or H_2SO_4 the modified van Slyke apparatus was difficult to clean due to masses of denatured protein on the glass walls.

With the limitations mentioned the deflection of the CO meter is practically proportionate to the volume of blood just as it is proportionate to the volume of a standard gas.

TABLE II The effect of the O_2 tension in the extraction chamber during the release of CO from blood 2 ml of blood were analyzed

Conditions compared for the release of CO from blood	n	CO content of the blood samples Range ml CO/100 ml blood	$\overline{D}$ ml CO/100 ml blood
$KH(JO_3)_2$			
Air—Vacuum	14	0 1—0.5	0 0088[2]
Air—N_2	11	0 1—0 4	0 0061
O_2—Vacuum	8	0 1—0 6	0 0086[2]
Preevacuation-vacuum	9	0 1—0 6	−0 0044
$K_3[Fe(CN)_6]$			
Air—Vacuum	9	0 1—0 3	0 0191[1]
Air—N_2	8	0 1—0 3	0 0042[1]
O_2—Vacuum	8	0 1—0 3	0 0153[2]
H_2SO_4			
Air—Vacuum	5	0 2—0 3	0 0123[1]
Preevacuation vacuum	5	0 2—0 6	−0 0039

$\overline{D}$ is the mean difference between the CO concentrations of paired blood samples extracted at two different O_2 tensions Other symbols as in Table I

Comparison between the different reagents

Blood samples of 1 or 2 ml with 0 07—0 34 ml CO/100 ml blood were analyzed with $K_3[Fe(CN)_6]$, H_2SO_4 and $KH(JO_3)_2$ With $KH(JO_3)_2$ slightly higher CO concentration was obtained than with H_2SO_4 and $K_3[Fe(CN)_6]$ (Table I), while there was no difference between the results with H_2SO_4 and $K_3[Fe(CN)_6]$ When 0 3 ml blood samples saturated with CO were analyzed with the different reagents the results did not differ statistically (Table I)

Some experiments were made which point to some possible errors with the different reagents due to chemical agents that may be used for cleaning of the extraction apparatus With $K_3[Fe(CN)_6]$ an extra amount of 6—7 μl CO/ml blood was obtained when 10 ml of a 10 per cent solution of NaOH was added to the blood reagent mixture With $KH(JO_3)_2$ only about 0 3 μl CO was obtained However, about 1 5—2 μl CO was also formed when the NaOH solution was added to 5 ml of the $K_3[Fe(CN)_6]$ reagent solution itself Using 5 ml of a solution containing 2 per cent H_2SO_4 and 5 per cent $K_2Cr_2O_7$ instead of a standard reagent solution resulted in an extra formation of about 10—15 μl CO/ml blood Similar results were obtained with the palladium molybdenum indicator tube method

TABLE III. Comparison between the method of Horvath and Roughton and the present one. Duplicate analyses were made by the two methods on the same blood sample.

	I. Method of Horvath and Roughton CO content ml CO/100 ml blood			II. The present method with $KH(JO_3)_2$ and 1 ml blood samples CO content ml CO/100 ml blood			Difference (I—II) ml CO/100 ml blood
n	Mean	S.D.	v	Mean	S.D.	v	
8	0.43	0.0[illegible]3	14	0.48	−0.004	0.[illegible]4	−0.05
10	[illegible].[illegible]9	0.033	10.5	0.9[illegible]	+0.02	2.89	−0.13
8	1.35	0.0[illegible]1	4.5	1.36	0.032	[illegible].35	0.01
12	[illegible].3	0.04	1	[illegible].30	0.0[illegible]1	2.64	+0.07

n = Number of duplicate analyses.
S.D. = Standard deviation calculated from duplicate analyses.
v = Coefficient of variation per cent.
For explanation of other symbols see Table I.

gas samples were successively transferred to the gas recipient. Fairly low O_2 partial pressures were obtained with the standard procedure (see Methods) and by filling the extraction chamber with CO-free N_2 gas. High partial pressures of O_2 were obtained by filling the extraction chamber with CO-free air or O_2. The results show that with all the reagents a slightly larger amount of CO was obtained when extraction was done in an atmosphere of air or O_2 than when it was done in N_2, in vacuum or in vacuum after preevacuation (Table II).

Standard procedure and precision of the method

The standard procedure for analysis of CO concentration on the basis of the above mentioned results was to use two vacuum extractions of 7 and 5 min. For low concentrations of CO in blood $K_3[Fe(CN)_6]$ or H_2SO_4 was used to release CO from the blood. $KH(JO_3)_2$ was often used at concentrations higher than about 0.5 ml CO/100 ml blood. The cleaning of the extraction chamber of the modified van Slyke apparatus was much easier after $KH(JO_3)_2$, and in this concentration range there was no significant difference between the results with $KH(JO_3)_2$ and with $K_3[Fe(CN)_6]$ or H_2SO_4.

The sensitivity of the CO meter should allow the determination of CO contents of about 0.005 ml CO/100 ml blood. The lowest CO concentration observed in blood treated with O_2 was 0.0[illegible] ml CO/100 ml. Several series of duplicate analyses on blood samples with a total amount of CO in the blood samples between 0.7 and [illegible]0 μl CO have shown the error to be fairly proportional to the amount of CO present in the analyzed blood sample. The coefficient of variation of a single determination was between 0.7—3 per cent in different series.

In the range of normal blood CO concentration (about 0.1 ml CO/100 ml blood) using a 2 ml sample the standard error of a single determination was about 0.002 ml CO/100 ml blood, and in measurements of the CO capacity of blood using 0.3 ml samples (about 0.5—0.8 ml CO/100 ml blood) the error was of the same magnitude with the reagents $K_3[Fe(CN)_6]$, H_2SO_4 and $KH(JO_3)_2$.

Comparison between the method of Horvath and Roughton and the present one

Comparisons between the method of Horvath and Roughton (1942) and the present one, using $KH(JO_3)_2$ as reagent were made on blood samples in two series (see Table III) In the lowest range in which the method described by Horvath and Roughton could be used it gave somewhat lower values than the present method The methodological error of Horvath and Roughton s method as calculated from duplicate determinations was large in this range while at a higher CO content the error decreased below that of the present method

Discussion

The method described allows determination of the low CO content in blood of normal subjects (COHb concentration about 0 5 per cent) and less with good reproducibility (about 3 per cent error) It also allows measurements of the high CO content of blood saturated with CO In this case the error of the method is larger than for instance, that of the method described by Horvath and Roughton (1942) The present method has been used by several investigators since it was first reported (Linderholm Sjostrand and Soderstrom 1957) and similar methodological errors have been found (Holmgren 1965 Bjure 1965)

An important question concerning the possibility of determining low COHb concentrations in blood is not related to the sensitivity of the analytical method but to the fact that CO may be formed in the breakdown of hemoglobin in vitro Sjöstrand (1951 1952 and 1953) thus found CO to be formed rapidly in blood at high temperature (38 C) and at low pH in the presence of such agents as ascorbic acid hydrogen peroxide and NaN_3 If CO is formed in the blood during the procedure of releasing CO from the hemoglobin with various chemical agents this may constitute an error in the determination of a low CO content of blood by such methods This question has been thoroughly analyzed by Coburn *et al* (1964) According to their results no CO production was to be expected during the release of CO from the blood by the present method using $K_3[Fe(CN)_6]$ If this supposition is correct our results would indicate that no CO is formed with H_2SO_4 either

The different results with various reagents and with various O_2 partial pressures in the extraction chamber show that slight changes in the conditions for the release of CO from the blood may be important Small amounts of CO were formed when O_2 of high partial pressure was present during the extraction procedure These amounts were so small however that they should only just be detectable by the method described by Coburn *et al* who did not find any difference if the CO was washed out by O_2 or by He CO production due to the presence of O should be of significance for the measurement of the CO content of blood only in the very low range of CO concentration and should be practically eliminated if extraction in vacuum or with N_2 is used

A slightly larger amount of CO was extracted on analysis with $KH(JO_3)_2$ than with $K_3[Fe(CN)_6]$ and H_2SO_4 Probably $KH(JO_3)_2$ causes extra CO production during the release of CO from blood due to an oxidation process that is enhanced by the stronger oxidizing power of $KH(JO_3)_2$ than $K_3[Fe(CN)_6]$ A high partial pressure of O_2 seems to have a similar effect

The small extra amount of CO obtained with $KH(JO_3)_2$ does not seem to cause a significant analytical error at concentrations higher than about 0 5 ml CO/100 ml blood $KH(JO_3)_2$ can therefore be used in this range with some advantage for the

release of CO from blood. The solution of $KH(JO_3)_2$ is quite stable and it was easy to clean the extraction apparatus when this reagent was used. Blank analysis must be checked, however.

H_2SO_4 and $K_3[Fe(CN)_6]$ seems to be equally useful for the release of CO from the blood in the case of a low CO content. $K_3[Fe(CN)_6]$ is not stable and CO may be released from this reagent with alkali. Together with blood and alkali a considerable amount of extra CO was formed, as has also been observed by Coburn *et al.* (1964). H_2SO_4 has the advantage of being stable and no traces of CO have been obtained with this reagent in blank analysis. The results show, however, that it is important to rinse the apparatus thoroughly after cleaning with chemical agents such as NaOH and $K_2Cr_2O_7$.

References

Bjurstedt, J. Pulmonary diffusing capacity for carbon monoxide in relation to cardiac output in man. *Scand. J. clin. Lab. Invest.* 1 17 Suppl. 81. 19[illegible]

Carlsten, A., A. Holmgren, K. Linroth, T. Sjöstrand and G. Ström. Relationship between low values of alveolar carbon monoxide concentration and carboxyhemoglobin percentage in human blood. *Acta physiol. scand.* 19[illegible] 31 6[illegible] 7[illegible]

Coburn, R. F., G. K. Danielson, W. S. Blakemore and R. E. Forster. Carbon monoxide in blood: analytical method and sources of error. *J. appl. Physiol.* 1964 19 510–51[illegible]

Falk, J. E. Porphyrins and metalloporphyrins. *Biochimica et Biophysica Acta Library* [illegible] 2. Elsevier Publ. Comp. Amsterdam, London, [illegible] New York 1964.

Gaensler, E. A., J. B. Jr. Cadigan, M. F. Ellicott, R. H. Jones and A. Marks. A new method for rapid precise determination of carbon monoxide in blood. *J. Lab. clin. Med.* 19[illegible] 4[illegible] 91[illegible] 9[illegible]7

Hartridge, H. Calibration of the reversion spectroscope for estimation of CO in blood. *J. Physiol. Lond.* 19[illegible] 57 47[illegible]

Holmgren, A. On the reproducibility of steady state D[illegible] measurements during exercise in man. *Scand. J. clin. Lab. Invest.* 19[illegible] 17 110–11[illegible]

Horvath, S. M. and F. J. W. Roughton. Improvements in gasometric estimation of carbon monoxide in blood. *J. biol. Chem.* 1942 144 747–[illegible]7

Kesston, [illegible] [illegible] M. Ellimann and A. [illegible] Spencer. The spectrophotometric determination of carbon monoxide. *J. biol. Chem.* 19[illegible] 183 2[illegible]7

Lawther, [illegible] J. and G. H. Apthorp. A method for the determination of carbon monoxide in blood. *Brit. J. industr. Med.* 19[illegible] 12 3[illegible]6 3[illegible]

Linderholm, H. A [illegible] method for the determination of carbon monoxide in blood. *Acta physiol. scand.* 1[illegible] [illegible] 3[illegible]2 3[illegible]

Linderholm, H. and T. Sjöstrand. Determination of carbon monoxide in small gas volumes. *Acta physiol. scand.* 1[illegible] [illegible] 21[illegible] 2[illegible]

Peters, [illegible] J. W. and W. [illegible] Ro[illegible]. The estimation of small amounts of carbon monoxide in blood. *J. biol. Chem.* 19[illegible] [illegible] 1[illegible]3 133

Scholander, P. F. and F. J. W. Roughton. Microgasometric estimation of blood gases. II. Carbon monoxide. *J. biol. Chem.* 1943 148 [illegible]1 [illegible]3

Sendroy, J. Jr. and S. H. Li[illegible]. Gasometric determination of oxygen and carbon monoxide in blood. *J. biol. Chem.* 1930 8[illegible] 133 [illegible]1

S[illegible], S. J. and T. Sjöstrand. A new method for determination of low concentrations of carbon monoxide in blood and the relation between the CO concentration in blood and that in the alveolar air. *Acta physiol. scand.* 19[illegible] [illegible] 1[illegible]

Sjöstrand, T. The [illegible] of carbon monoxide in blood. *Acta physiol. scand.* 19[illegible] 2[illegible] 31[illegible] 33[illegible]

Sjöstrand, T. The formation of carbon monoxide by [illegible] decomposition of haemoglobin in vivo [illegible] pigments. *Acta physiol. scand.* 1[illegible] [illegible] 3[illegible]8 333

Sjöstrand, T. The formation of carbon monoxide in the presence of NaN_3. *Acta physiol. scand.* 19[illegible]3 2[illegible] 21[illegible] 2[illegible]

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From the Department of Physiological Chemistry, University of Lund Sweden

The Effect of Glucose Availability and Utilization on Chylomicron Metabolism in the Rat

By

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Abstract

Brown D F and T Olivecrona *The effect of glucose availability and utilization on chylomicron metabolism in the rat* Acta physiol scand 1966 *66* 9—18 — Following i v administration [illegible]

uptake of triglyceride fatty acid might be related to the availability of α glycerophosphate for esterification it is equally possible that adipose tissue lipoprotein lipase content may be a regulating factor Heparin as opposed to saline administration prior to chyle injection produced marked acceleration in the rate of removal of chyle from the blood of treated and untreated diabetic animals The accelerated removal was related to accelerated uptake of activity by the liver and could be attributed in part to intravascular lipolysis of chyle triglyceride The effect of heparin on the degree and the nature of hepatic uptake was no different in treated and untreated diabetic animals

Abnormal lipid levels in man might be related to abnormal glucose metabolism Elevated serum triglyceride levels have been reported in diabetes mellitus (New *et al* 1963), and diabetics are said to exhibit prolonged alimentary lipemia after a fat meal (Berkowitz 1962)

Albrink (1957) has demonstrated that the degree of alimentary lipemia after a fat meal is less if the same amount of fat is fed with glucose and has suggested that the availability of glucose may accelerate the uptake of triglyceride by adipose tissue This hypothesis bears some support from experimental studies It has been shown that the triglyceride molecule does not enter adipose tissue intact but that the fatty acid component enters only after lipolysis of the triglyceride has occurred (Shapiro, Chowers and Rose 1957)

Although glycerol is produced by lipolysis of adipose tissue triglyceride this is largely or completely unavailable for re-esterification due to lack of glycerol kinase (Vaughan 1961). An alternative source of glycerol is thus required to allow fatty acid entering adipose tissue to be esterified and stored as triglyceride. There is ample evidence that this esterification is dependent on an adequate supply of L-α-glycerophosphate derived from glucose. It is not unexpected therefore that the incorporation of triglyceride fatty acid and free fatty acid into adipose triglyceride should be dependent on glucose availability.

In vivo studies (Bragdon and Gordon 1958) (Havel, Felts and Van Duyne 1962) and in vitro studies (Bezman, Felts and Havel 1962) have shown that triglyceride fatty acid incorporation into adipose tissue is greater in refed than in fasted animals. No information is available on the metabolism in vivo of chylomicrons in diabetes mellitus nor on the effect in vivo of insulin on chylomicron metabolism. This study was carried out to provide this information by measuring the tissue distribution of H³ palmitic acid labelled chylomicrons in Alloxan-diabetic rats. A comparison was made of the effect of modifying glucose availability by fasting and refeeding with that produced by insulin withdrawal or administration in diabetes mellitus.

As triglyceride uptake by adipose tissue is dependent on prior lipolysis, presumably by lipoprotein lipase and as this enzyme is low in concentration in adipose tissue from fasted (Cherkes and Gordon 1959) and from diabetic rats (Schnatz and Williams 1963) the effect of heparin liberated lipoprotein lipase on chylomicron metabolism also was studied in treated and untreated diabetic rats.

Methods

Preparation of Chylomicrons

[illegible] (9,10-H³ palmitic acid [illegible]) was neutralized with ethanolic potassium hydroxide and soaked up in a small piece of bread. After evaporating the alcohol the bread was fed to a male Sprague Dawley rat on which a thoracic duct cannulation had been performed 12-24 hrs previously. Approximately 3 g of butter were fed with or immediately after the isotope. The rat was allowed to continue drinking a solution containing 0.4 g of NaCl and 0.4 g KCl in water. Chyle [illegible] commenced following the thoracic duct cannulation. Chyle was collected in a dry Erlenmeyer flask at room temperature and allowed to clot. The fibrin was removed with a glass rod. The chyle was stored at [illegible] C and used within 24 hrs of its collection after first being allowed to reach room temperature.

Depending on its activity 0.5 to 1.0 ml of chyle ([illegible] 8 [illegible] 10⁶ cpm) was injected into the exposed [illegible] jugular vein of animals studied. With the exception of experiment B in which 6.2 mg of lipid was injected the amount of injected lipid varied from 18-26 mg. Separation of chyle lipids by thin layer chromatography and measurement of radioactivity in the eluted fractions (Brown and [illegible] 196[illegible]) revealed that more than 97 % of the activity was located in triglycerides. More than 8[illegible] of blood radioactivity was present in the triglyceride fraction except in animals treated with heparin.

Injection of Chyle and Recovery from Tissue

Animals were lightly anesthetized with ether prior to chyle injection and then allowed to regain consciousness. Seven minutes later they were reanesthetized and 10 min after chyle injection were exsanguinated via the aorta. The blood was transferred into preweighed centrifuge tubes containing a few drops of heparin [illegible] and weighed. It was then transferred to a Hagedorn tube containing [illegible] ml of ethanol. After mixing exactly 10 ml of chloroform was added and followed immediately by [illegible] saline. The tube was allowed to stand in the cold overnight and in the morning 8 ml of the chloroform phase removed (Carlson 1963). Exactly [illegible] containing blood lipids from 0.1 ml blood was taken into a scintillation vial and evaporated prior to the addition of 10 ml of toluene containing 3 g 2,5-diphenyloxazole and 100 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene per litre. The radioactivity was then measured in a Packard

sodium sulphate, filtered into a 250 ml volumetric flask and made up to volume with chloroform After mixing thoroughly, 10 ml was transferred to a scintillation vial, the chloroform evaporated and the lipid activity counted

After removing the liver the rat was skinned The skin and carcass were separately digested in 100 and 500 ml of alcoholic potassium hydroxide respectively (20 g KOH, 20 ml water 80 ml

radioactivity counted

These procedures permitted the calculation of radioactivity in liver skin carcass and blood

in subcutaneous adipose tissue was measured directly, as it is relatively homogenous and forms the major part of total adipose tissue it was decided that it would serve as the best index of adipose tissue activity under the condition of this study

The amount of blood remaining in the carcass was calculated by subtracting from total blood volume the amount of blood withdrawn from the aorta The calculated blood radioactivity in the carcass was then subtracted from the total activity in the carcass digest and the resulting figure taken as the true carcass activity

In each e-
60 ml of 2
equilibratin
and made u

activity counted to yield duplicate values for the amount of radioactivity injected. The total activity in blood and tissues is expressed as percent of injected activity Male Sprague Dawley rats were used throughout Blood sugar levels were measured using the method of Cerotti (1963)

Experiment A Chylomicron Metabolism in Fasted and in Refed Rats

The tissue distribution of chylomicron fatty acid was studied in healthy fasted and healthy

Experiment B The Acute Effect of Glucose on Chylomicron Metabolism

The effect of short term administration of glucose on chylomicron metabolism was studied after

Blood glucose levels were determined at death.

TABLE I. The tissue distribution of label in 9 fasted and in 9 refed rats 10 min after the i.v. injection of chylomicrons labelled with H^3 palmitic acid. Values expressed as percent of injected radioactivity

	Weight (g)	Blood	Liver	Skin	Carcass
Fasted	176.1 ± 23.4	33.6 ± 7.7	18.6 ± 4.0	3.9 ± 0.8	13.4 ± 5.5
Refed	180.6 ± 18.6	22.9 ± 12.5	24.1 ± 7.5	6.2 ± 1.7	23.3 ± 10.0
P Value	N.S.	< 0.05	N.S.	< 0.01	< 0.02

After alloxan injection the rats were allowed free access to food. 1—3 days later glycosuria was detected in the majority of animals and fasting blood sugars were elevated.

For experiment C 3 diabetic animals were used 1 week after diabetes became evident. They received no insulin.

These 3 diabetic rats and 6 healthy rats were fasted for 24 hrs, allowed free access to 20 % glucose in half strength saline for 24 hrs and then injected with chyle. 2 hrs and again 5 min before chyle injection 3 of the healthy animals were given 6 and 4 units respectively of crystalline insulin by i.v. injection. All animals were killed 10 min after chyle injection and processed in the usual fashion.

Experiment D. Chylomicron Metabolism in Insulin Treated and Insulin Deprived Alloxan-Diabetic Rats

When it became apparent that the diabetic rats rapidly lost weight and had a high mortality rate after fasting it was decided to treat them with protamine zinc insulin (PZI). In this way it was possible to maintain a diabetic colony indefinitely and this proved more convenient for future experiments. The severity of the diabetes could be varied according to length of time insulin was withheld. 8 insulin maintained diabetic rats matched for weight were removed from the colony, but allowed free access to food and water. Insulin was discontinued in 4 animals. 50 hrs later they were injected with chyle and the tissue distribution measured. In the other 4 animals the usual dose of 6 units PZI insulin was given 24 hrs after their removal from the colony. On the following day PZI was withheld and 6 units of crystalline insulin given s.c. 4 hrs and again 1/2 hr prior to chyle injection at 50 hrs. Insulin treated and insulin deprived animals were injected with chyle alternately and then handled in the usual fashion.

Experiment E. The Effect of Heparin on Chylomicron Metabolism in Insulin Treated and Insulin Deprived Diabetic Rats

20 diabetic animals were removed from the colony and 10 deprived of insulin for 36 hrs. On the evening that chyle was injected the other 10 animals received 6 units of crystalline insulin s.c. 2 hrs before chyle injection. At 8 a.m. of that morning their usual dose of PZI was replaced by a smaller dose of 4 units. All animals had free access to food and water.

5 of the insulin treated animals were given 0.5 ml saline and 5 were given 2.0 mg heparin/kg by i.v. injection, 1 min before chyle injection. The insulin deprived animals were treated in the same manner.

Results

Experiment A. Chylomicron Metabolism in Fasted and in Refed Rats

The tissue distribution of injected chyle radioactivity in fasted and in refed rats is shown in Table I. The mean value for the amount of activity remaining in blood was significantly higher in the fasted animals. A significantly greater amount of radioactivity was found in the adipose tissue and carcass of refed animals. This difference was found when the activity was expressed either as a percent of the injected dose as in Table I or as a percent of the total activity cleared from blood. Although the mean level for

TABLE II The tissue distribution of chylomicron palmitic acid (H^3 labelled) in 11 fasted rats 90 min after intraperitoneal injection of 2 ml saline or 2 ml 30% glucose

	Weight (g)	Blood sugar mg %	Per cent activity in blood	Per cent activity in liver	Per cent activity in skin	Per cent activity in carcass
Gluc [1]	173 3±2 6	134 2±14 6	43 3±12 8	19 4±7 8	4 1±0 8	15 2±4 2
Sal [2]	179 0±11 4	84 8±7 9	27 5±1 9	19 7±0 9	4 4±0 7	16 3±3 9
P Value	N S S	P<0 001	P 0 025	N S S	N S S	N S S

Gluc [1] = Glucose
Sal [2] = Saline

TABLE III The tissue distribution of chylomicron palmitic acid (H^3 labelled) in 10 fasted rats after a 1 hr i v infusion with 2 ml saline or 2 ml 30% glucose

	Weight (g)	Blood sugar mg %	Per cent activity in blood	Per cent activity in liver	Per cent activity in skin
Glucose	185 0+6 1	350 3±67 0	41 0±10 5	12 0±2 7	3 8±0 5
Saline	184 4±8 0	86 0±6 9	39 0±8 8	13 6±2 3	3 7±0 3
P Value	N S S	P<0 001	N S S	N S S	N S S

hepatic radioactivity was higher in the refed than in the fasted animal the difference was not statistically significant The total amount of radioactivity recovered was less in the fasted than in the refed animals In the fasted group a mean of 31 % and in the refed 23 % of injected activity was not recovered and is thought to represent fatty acid oxidation These findings are consistent with the sparing of fatty acid oxidation by glucose

[illegible]

thought to represent the sparing of fatty acid oxidation by glucose

Although the i v glucose produced marked elevation of blood sugar levels no difference in the tissue distribution of radioactivity was found between glucose and saline infused animals (Table III)

When the amount of lipid injected in these two experiments was calculated after completion of the experiment it was found that 62 mg of lipid was injected as compared with 18 mg in exp A This larger dose might account for the large amount of activity remaining in the blood of these animals

TABLE IV A comparison of the tissue distribution of chylomicron palmitic acid (H³ labelled) in alloxan diabetic refed rats normal refed rats and normal refed rats treated with insulin

Clinical state	Rat #	Weight (g)	Blood sugar mg %	Blood	Liver	Skin
Diabetes	169	145	466	22 0	33 7	2 5
	170	160	485	17 5	30 0	2 1
	171	135	478	24 9	41 0	2 5
	Mean	146 7	476 3	21 5	34 9	2 4
Normal	184	235	170	22 4	26 8	3 3
	185	255	160	24 5	45 6	3 8
	186	240	162	27 2	43 9	3 1
	Mean	243 3	164 0	24 7	38 8	3 4
Insulin in normal rats	173	240	47	25 2	26 0	4 7
	174	250	66	17 8	30 4	7 6
	175	230	65	31 0	9 8	8 3
	Mean	240 0	59 3	24 7	25 4	6 9

TABLE V The mean tissue distribution of chylomicron palmitic acid (H³ labelled) in 4 insulin treated (A) and 4 insulin deprived (B) alloxan-diabetic rats

Weight (g)	Blood sugar mg %	Per cent activity in blood	Per cent activity in liver	Per cent activity in carcass	Per cent activity in skin	Specific activity skin lipid cpm/mg
A—208 8 ± 14 4	77 8 ± 25 1	13 0 ± 2 6	33 0 ± 4 9	19 6 ± 6 6	9 7 ± 3 1	207 0 ± 39 3
B—182 5 ± 25 3	398 8 ± 17 8	17 1 ± 5 6	37 3 ± 6 8	15 7 ± 4 8	1 9 ± 0 3	56 0 ± 12 9
P Value NSS	P 0 001	NSS	NSS	NSS	P < 0 005	P 0 001

Experiment C A Comparison of Chylomicron Metabolism in the Healthy Refed Rat in the Healthy Refed Rat Treated With Insulin and in the Insulin Deficient Rat Table IV lists the blood sugar levels and tissue distribution of radioactivity in animals described in exp C The diabetic animals exhibited severe hyperglycemia and a consistently low uptake of activity into skin Insulin a[illegible] to normal animals produced considerably lower blood sugar levels t[illegible] noninsulin treated controls. The adipose tissue activ[illegible] of [illegible] ar[illegible] than that of the diabetic animals and was greatr[illegible] [illegible] imals

TABLE VI The effect of heparin on the tissue distribution of chylomicron palmitic acid in 5 insulin treated diabetic rats

	Weight (g)	Blood sugar mg %	Per cent activity in blood	Per cent activity in liver	Per cent activity in skin
Sal [1]	193 0±29 7	173 6±102 4	20 3±9 5	18 8±6 1	10 6±2 6
Hep [2]	208 0±14 0	181 6±21 8	5 1±1 5	42 4±3 4	4 7±0 6
P Value	N S S	N S S	P< 0 02	P< 0 001	P< 0 005

Sal [1] = Saline injected
Hep [2] = Heparin injected

TABLE VII The effect of heparin on the tissue distribution of chylomicron palmitic acid in five insulin deprived diabetic rats

	Weight (g)	Blood sugar mg %	Per cent activity in blood	Per cent activity in liver	Per cent activity in skin
Sal [1]	177 0±22 0	355 0±69 0	32 1±8 9	19 3±1 6	5 7±2 9
Hep [2]	177 0±9 7	320 0±23 7	5 0±0 6	44 5±3 4	3 3±0 5
P Value	N S S	N S S	P< 0 001	P< 0 001	P 0 10

Sal [1] Saline injected
Hep [2] Heparine injected

Experiment D Chylomicron Metabolism in Insulin Treated and Insulin Deprived Alloxan Diabetic Rats

Differences in adipose tissue uptake as seen in exp C but of greater magnitude were seen in insulin maintained and insulin deprived diabetic animals (Table V)

The specific activity of adipose tissue from the former was almost 4 times that of the latter Although 2 of the insulin deprived animals exhibited high levels of activity in blood the other two did not differ from the insulin treated animals The mean value for hepatic uptake was higher in the insulin deprived animals

Experiment E The Effect of Heparin on Chylomicron Metabolism in Insulin Treated and Insulin Deprived Diabetic Rats

In this final experiment in which insulin was withheld for 36 hrs as compared with 50 hrs in exp D a surprisingly large amount of activity was recovered in the skin of insulin deprived saline treated animals inspite of considerably elevated blood sugar levels Nevertheless the mean adipose tissues activity in this group was significantly lower (P = 0 025) than that of the insulin treated control group

TABLE IV A comparison of the tissue distribution of chylomicron palmitic acid (H^3 labelled) in alloxan-diabetic refed rats, normal refed rats and normal refed rats treated with insulin

Clinical state	Rat #	*Weight* (g)	*Blood sugar* mg %	Blood	Liver	Skin
Diabetes	169	145	466	22 0	33 7	2 5
	170	160	485	17 5	30 0	2 1
	171	135	478	24 9	41 0	2 5
	Mean	146 7	476 3	21 5	34 9	2 4
Normal	184	235	170	22 4	26 8	3 3
	185	255	160	24 5	45 6	3 8
	186	240	162	27 2	43 9	3 1
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Insulin in normal rats	173	240	47	25 2	26 0	4 7
	174	250	66	17 8	30 4	7 6
	175	230	65	31 0	9 8	8 3
	Mean	240 0	59 3	24 7	25 4	6 9

TABLE V The mean tissue distribution of chylomicron palmitic acid (H^3 labelled) in 4 insulin treated (A) and 4 insulin-deprived (B) alloxan diabetic rats

Weight (g)	Blood sugar mg %	Per cent activity in blood	Per cent activity in liver	Per cent activity in carcass	Per cent activity in skin	Specific activity skin lipid cpm/mg
A—208 8 ± 14 4	77 8 ± 25 1	13 0 ± 2 6	33 0 ± 4 9	19 6 ± 6 6	9 7 ± 3 1	207 0 ± 39 3
B—182 5 ± 25 3	393 8 ± 17 8	17 1 ± 5 6	37 3 ± 6 8	15 7 ± 4 8	1 9 ± 0 3	56 0 ± 12 9
P Value-N S S	P 0 001	N S S	N S S	N S S	P< 0 005	P=0 001

Experiment C A Comparison of Chylomicron Metabolism in the Healthy Refed Rat, in the Healthy Refed Rat Treated With Insulin and in the Insulin Deficient Rat Table IV lists the blood sugar levels and tissue distribution of radioactivity in animals described in exp C The diabetic animals exhibited severe hyperglycemia and a consistently low uptake of activity into skin Insulin administration to normal animals produced considerably lower blood sugar levels than were found in noninsulin treated controls The adipose tissue activity of normal animals was greater than that of the diabetic animals and was greater in insulin treated than in nontreated normal animals

after cessation of insulin injections to alloxan-diabetic rats The possibility must be considered therefore that adipose tissue lipoprotein lipase levels rather than glucose availability might be the primary mechanism regulating the entry of triglyceride fatty acid into this tissue and that the availability of glucose is fortuitous This would receive some support from the finding that although adipose tissue uptake of triglyceride fatty acid is influenced by the nutritional state the uptake of albumin bound fatty acid is not (Bezman, Felts and Havel 1962) It is possible that the failure of intraperitoneal or intravenous glucose administration to fasted animals to influence the incorporation of triglyceride fatty acid into adipose tissues might be related to the length of time required to replete adipose tissue lipoprotein lipase levels In this study it was evident that even in the presence of diabetes, heparin was able to produce accelerated clearing of triglyceride Hollenberg (1960) has demonstrated that in starvation, heart lipoprotein lipase is increased while adipose tissue level is decreased It is probable that tissues other than adipose were responsible for the liberation of lipoprotein lipase by heparin in the diabetic rats of this study It was noteworthy that hepatic uptake of triglyceride activity was significantly higher and adipose tissue uptake lower in heparin treated than in saline treated animals Morris and French (1958) have shown that preincubation of chylomicrons with clearing factor produces accelerated hepatic uptake

More recent work from this laboratory suggests that the greater hepatic uptake of activity in the heparin treated animals can be related to the fact that while the entry of intact triglyceride occurs at the same rate as in saline treated controls it is also accompanied by the simultaneous uptake of free fatty acid produced in the blood from the effect of heparin liberated lipoprotein lipase on the injected chyle

This work was carried out during tenure of a fellowship to D F Brown from the National Heart Institute HSP 16 647 He expresses his gratitude to Professor B. Borgstrom for providing laboratory space and facilities to carry out this work

References

ALBRINK M J and E B MAN Effect of carbohydrate ingestion on postprandial lipemia *Amer J dig Dis* 1957 *2* 649—653

BERKOWITZ D Serum lipid and fat tolerance determinations in controlled diabetics *Diabetes* 1962 *11* Suppl 56—61

BEZMAN A J M FELTS and R J HAVEL. Relation between incorporation of triglyceride fatty acids and heparin released lipoprotein lipase from adipose tissue slices *J Lipid Res* 1962 *3* 427—431

BI[illegible]

BF[illegible]

37 574—578

BROWN J L. and J M JOHNSTON Radio assay of lipid components separated by thin layer [illegible]

F[illegible]

F[illegible]

F[illegible]
diaphragm *J clin Invest* [illegible]

KESSLER J I, Effect of insulin on release of plasma lipolytic activity and clearing of emulsified fat intravenously administered to pancreatectomized and alloxanized dogs *J Lab clin Med* 1962 *60* 747—755
MORRIS B and J E FRENCH, The uptake and metabolism of ^{14}C labelled chylomicron fat by the isolated perfused liver of the rat *Quart J exp Physiol* 1958 *43* 180—188
NEW MARIA I T N ROBERTS, E L BIERMAN and G G READER, The significance of blood lipid alterations in diabetes mellitus *Diabetes* 1963 *12* 208—212
OLIVECRONA T, E P GEORGE and B BORGSTRÖM Chylomicron metabolism *Fed Proc* 1961 *20* 928—933
SCHNATZ J D and R H WILLIAMS The effect of acute insulin deficiency in the rat on adipose tissue lipolytic activity and plasma lipids *Diabetes* 1963 *12* 174—178
SHAPIRO B I CHOWERS and G ROSE Fatty acid uptake and esterification in adipose tissue *Biochim biophys Acta (Amst)* 1957 *23* 115—120
VAUGHAN M, The metabolism of adipose tissue in vitro *J Lipid Res* 1961 *2* 293—316

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From the Department of Physiology, University of Goteborg Sweden

An Evoked Potential Study of Different Pathways from the Hindlimb to the Somatosensory Areas in the Cat

By

Ulf Norrsell and Edward R. Wolpow[1]

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Abstract

The dorsal columns and the medial lemniscus have for a long time been considered to be part of one of the major routes for somatosensory impulses to the cerebral receiving areas However, short latency evoked potentials can be elicited in the cerebral somatosensory areas after transection of the dorsal columns (Gardner and Noer 1952 Gardner and Haddad 1953, Morin 1955, Catalano and Lamarche 1957, Mark [illegible]

[illegible]

spindle afferents from the forelimb (Oscarsson and Rosén 1963 [illegible] low threshold muscle

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The large potentials evoked by stimulation of cutaneous nerves after transection of the dorsal columns are conducted via a pathway in the dorsal part of the lateral funiculus which crosses over to the contralateral ventral funicle at the C_1—C_3 level presumably after a relay in the lateral cervical nucleus (Morin 1955, Busch 1961, Norrsell and Voorhoeve 1962) The pathway responsible for the conducion to the upper cervical segments is the spinocervical tract (Norrsell and Voorhoeve 1962, Lundberg 1964) The cell bodies of this tract are located in the dorsal horn in monosynaptic contact with ipsilateral primary afferents directly as they enter and the axons ascend in the dorsomedial part of the lateral funicle and terminate in the upper cervical region (Lundberg and Oscarsson 1961, Lundberg 1964)

However, the spinal pathways responsible for the cortical potentials evoked from other somatic afferents, e g the high threshold muscle and joint afferents, are still little known Thus in the present investigation different types of peripheral nerves of the cat's hindlimb were stimulated electrically and evoked potentials recorded in the contralateral somatosensory areas before and after various discrete lesions of the spinal cord

Methods

The experiments were peformed on adult cats anesthetized with pentobarbitone sodium (Veterinary Nembutal Abbott) and paralyzed with gallamonium triethiodide (Flaxedil May & Baker Ltd) The animals were slightly hyperventilated with 94 % O_2 and 6 % CO_2 When necessary low molecular dextrane (Rheo-Macrodex Pharmacia) was given by slow i v infus on

A one sided cran ectomy was made and the dura removed over an area large enough to give easy access to the posterior sigmoid gyrus and the anterior ectosylvian gyrus of that side The spinal cord was exposed in the lower thoracic segments in order to make the lesions and in segments L_7—S_1 for recording the incoming nerve volley at the dorsal root entry zone Nerves of the hindlimb opposite to the craniectomy were dissected and their distal ends cut The dissected nerves were the hamstring nerve (n m biceps n m semitendinosus and n m semimembranosus) n suralis n peroneus superficialis (deprived of n m peroneus longus and n m peroneus tertius) n m gastrocnemius soleus the posterior joint nerve and the nerve to the interosseous membrane (branching off the n tibialis together with the branches to the flexor longus digitorum muscle and dissected free from these) All other nerves of both hindlimbs were cut All exposed tissues were covered with mineral oil kept at 38° C

At the beginning of each experiment the cortical surface was explored with the record ng electrode to determine the points of maximum response for the various nerves The locations corresponded well to the hindlimb projection areas in SI and SII described by Woolsey (1947) The threshold at which a cortical potential could be evoked from a particular nerve was deter [illegible]

formed and the procedure repeated for the third time — Since the results could be obscured by dorsal root reflexes we were always careful to keep the body temperature of the experimental animals at 38 C throughout the experiments For control we recorded from each of the dissected peripheral nerves looking for dorsal root reflexes while recording the evoked potentials simultaneously In no instance were we able to attribute an evoked potential to a dorsal root reflex

Ag AgCl electrodes were used and the cortical electrode was springmounted and with a ball end The stimuli consisted of condenser discharges with a 45 μsec half time decay delivered at 1 sec intervals. The stimulus intensity was graded in multiples of the threshold stimulus strength for the nerve The evoked potentials were recorded with one electrode on the cortical surface against a reference electrode in the temporal muscle The lesions were made with fine watchmaker s forceps under a binocular dissecting microscope and special care was always taken to avoid damaging blood vessels After each experiment the lesioned part of the spinal

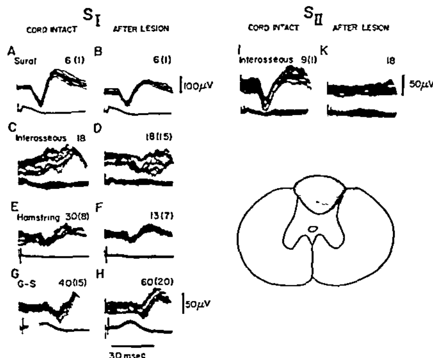


Fig 1 Superimposed records of cortical potentials, upper traces, elicited before and afte transection of the dorsal columns The records to the left in each double row were obtained before and those to the right after the lesion was made The left double row depicts potentials elicited in the contralateral S_I and the right double row depicts potentials elicited in the contralateral S_{II} [illegible]

cortical records situated to the left of it and above

cord was removed and fixed in 10 per cent formalin It was then mounted in paraffin and examined unstained in 50 μ sections.

DLF denotes the dorsal part of the lateral funiculus G-S the gastrocnemius-soleus nerve and SP the superficial peroneal nerve The expressions ipsilateral and contralateral refer to the stimulated nerves in all cases where no specific reference has been given

Results

Evoked potentials recorded with the spinal cord intact

The results of recording evoked potentials in cats with the spinal cord intact are summarized in Tables I (S_I) and II (S_{II})

Cutaneous nerves The largest and most regularly evoked cortical potentials were those from cutaneous nerves The average latencies were similar for the potentials from both nerves used even though the conduction distance from the SP nerve averaged 50 mm longer The amplitude of the cutaneous evoked potentials did not show any consistent

The large potentials evoked by stimulation of cutaneous nerves after transection of the dorsal columns are conducted via a pathway in the dorsal part of the lateral funiculus which crosses over to the contralateral ventral funicle at the C_1—C_2 level presumably after a relay in the lateral cervical nucleus (Morin 1955, Busch 1961, Norrsell and Voorhoeve 1962). The pathway responsible for the conducion to the upper cervical segments is the spinocervical tract (Norrsell and Voorhoeve 1962, Lundberg 1964). The cell bodies of this tract are located in the dorsal horn in monosynaptic contact with ipsilateral primary afferents directly as they enter and the axons ascend in the dorsomedial part of the lateral funicle and terminate in the upper cervical region (Lundberg and Oscarsson 1961, Lundberg 1964).

However, the spinal pathways responsible for the cortical potentials evoked from other somatic afferents, e.g. the high threshold muscle and joint afferents, are still little known. Thus in the present investigation different types of peripheral nerves of the cat's hindlimb were stimulated electrically and evoked potentials recorded in the contralateral somatosensory areas before and after various discrete lesions of the spinal cord.

Methods

The experiments were peformed on adult cats anesthetized with pentobarbitone sodium (Veterinary Nembutal Abbott) and paralyzed with gallamonium triethiodide (Flaxedil May & Baker Ltd). The animals were slightly hyperventilated with 94 % O_2 and 6 % CO_2. When necessary low molecular dextrane (Rheo-Macrodex, Pharmacia) was given by slow i.v. infusion.

ed nerves were the hamstring nerve (n.m. biceps, n.m. semitendinosus and n.m. semimembranosus), n. suralis, n. peroneus superficialis, deprived of n.m. peroneus longus and n.m. peroneus tertius, n.m. gastrocnemius-soleus, the posterior joint nerve and the nerve to the interosseous membrane (branching off the n. tibialis together with the branches to the flexor longus digitorum muscle and dissected free from these. All other nerves of both hindlimbs were cut. All exposed tissues were covered with mineral oil kept at 38° C.

At the beginning of each experiment the cortical surface was explored with the recording electrode to determine the points of maximum response for the various nerves. The locations corresponded well to the hindlimb projection areas in SI and SII described by Woolsey (1947). The threshold at which a cortical potential could be evoked from a particular nerve was determined at the same time as the mapping was performed. Lesions were then made. Either the dorsal columns or the dorsal part of the lateral funiculus (DLF, see drawing Fig. 3) were transected at the low thoracic level. The mapping and the threshold determinations were then repeated. After this the one of the two above mentioned lesions not made previously was performed and the procedure repeated for the third time. — Since the results could be obscured by dorsal root reflexes we were always careful to keep the body temperature of the experimental animals at 38° C throughout the experiments. For control we recorded from each of the dissected peripheral nerves looking for dorsal root reflexes while recording the evoked potentials simultaneously. In no instance were we able to attribute an evoked potential to a dorsal root reflex.

Ag-AgCl electrodes were used and the cortical electrode was springmounted and with a ball end. The stimuli consisted of condenser discharges with a 45 μsec half time decay delivered at 1 sec intervals. The stimulus intensity was graded in multiples of the threshold stimulus strength for the nerve. The evoked potentials were recorded with one electrode on the cortical surface against a reference electrode in the temporal muscle. The lesions were made with fine watchmaker's forceps under a binocular dissecting microscope and special care was always taken to avoid damaging blood vessels. After each experiment the lesioned part of the spinal

TABLE I Average latencies (with standard deviations) and thresholds of all[1] evoked potentials recorded in S_I with the spinal cord intact

Nerve	Latency from the shock artefact to the onset of the initial positivity msec	Threshold in multiples of nerve threshold	No of experiments from which averages were calculated	Response from nerve obtained in % of experiments	Illustrated in figure
Sural	9 2±0 9	1–2	10	59	1 A
Superficial peroneal	9 5±1 2	1–2	14	87	4 A
Posterior joint	16 8±3 0	2 5–8	6	40	2 A and 4 C
Hamstring[1] 'early' group	11 6±1 5	1 3–8	8	47	1 E 2 C and 3 A
late group	26 0±3 3	10	3	18	
Gastrocnemius-soleus	19 1±0 4	9	5	29	1 G and 3 C
Interosseous	15 9±3 4	2–4	5	31	1 C and 4 E

[1] One potential evoked from the hamstring nerve at a strength 15 times the threshold strength for the nerve and with a latency of 13 5 msec has not been included in the calculations

TABLE II Average latencies (with standard deviations) and thresholds of all evoked potentials recorded in S_{II} with the spinal cord intact

Nerve	Latency from the shock artefact to the onset of the initial positivity msec	Threshold in multiples of nerve threshold	No of experiments from which averages were calculated	Response from nerve obtained in % of experiments	Illustrated in figure
Sural	10 8±2 4	1–2	13	76	
Superficial peroneal	9 8±1 3	1–2	16	94	2 E and 4 G
Posterior joint	16 7±2 8	2 5–8	6	35	2 G and 3 E
Hamstring early group	13 5±2 9	3–5	4	24	2 L and 3 G
late group	22 8±4 4	8	5	29	
Gastrocnemius-soleus early group	12 0±0 8	2–3	3	18	
late group	22 2±3 6	9	11	65	2 L and 3 I
Interosseous	12 0±1 7	2	15	94	1 I and 4 I

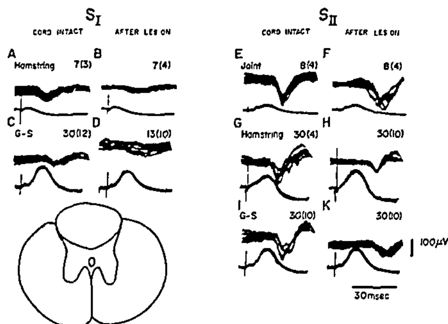


Fig 3 Potentials evoked in the hindlimb regions of the right somatosensory areas from left hindlimb nerves before and after transection of the left DLF Arrangement and symbols as in Fig 1

of the interosseous nerve In that experiment an interosseous evoked potential could be elicited in S_{II} at the usual strength i e less than twice the threshold of the nerve

The joint nerve Evoked potentials from the posterior joint nerve were recorded in both S_I and S_{II} in 5 expts out of 15 In 2 more expts they were found in either area but 60 per cent of the experiments gave negative results The latencies of these potentials varied considerably between different experiments in either area as evinced from the standard deviations given in tables The joint evoked potentials recorded in S_I (Fig 2, A and 4 C) were always of small amplitude whereas those recorded in S_{II} (Fig 2, G and 3, E) in some instances attained an amplitude comparable to that of the cutaneous evoked potentials The joint evoked potentials were never seen to develop simultaneously with the two first components of the afferent neurogram (cf Skoglund 1956) but appeared at stimulus intensities 2 5—8 times the threshold of the nerve

Muscle nerves Stimuli to either of the two muscle nerves gave rise to evoked potentials in both somatosensory areas (see Table I and II) These potentials, however, formed two groups with different characteristics One group labelled "early" in the tables was elicited at lower stimulus intensity and had shorter latency whereas the potentials labelled "late" in the tables were elicited at higher stimulation intensity and had longer latency

The "early" muscle evoked potentials recorded in S_I were in all instances elicited from the hamstring nerve (Fig 1 F, Fig 2, C and Fig 3, A) The threshold of these varied from 1 3—8 times the threshold of the nerve Thus in two experiments the hamstring "early" evoked potential was seen to develop before the group I spike had attained its maximum but in all the experiments it had started to develop before group III

TABLE III Latencies and differences from pre-lesion latencies of all evoked potentials recorded in both somatosensory areas after transection of the dorsal columns[1]

Nerve	Experiment	Recorded in S_I Latency msec	Difference from prelesion value msec	Illustrated in figure	Recorded in S_{II} Latency msec	Difference from prelesion value msec	Illustrated in figure
Sural	1	9.5	0	1, B	11.5	0	
	2	—			12.5	−0.5	
	3	10.0	+1.5		11.5	+2.0	
	4	9.0	0		—		
Superficial peroneal	1	10.0	0		10.0	0	
	2	10.0	+0.5		10.5	+0.5	
	3	10.5	+1.5		11.0	0	2 F
	4	9.0	0		11.0	0	
Posterior joint	1	19.0	+1.0		19.0	+1.0	
	2	12.0	0		—		
	3	20.0	0	2 B	19.0	+1.5	2, H
Hamstring early group	1	15.0	+1.0	1 F			
	2	13.0	+2.5				
	3	14.0	+2.0	2 D	14.0	0	2, K
late group	1	—			18.5	+1.5	
	2	gone			gone		
	3	gone			—		
Gastrocnemius soleus late group	1	20.0	+0.5	1 H	22.0	−1.0	
	2	gone			gone		
	3	20.0	+1.5		20.0	+0.5	2 M
Interosseous	1	22.0	+0.5	1 D	gone		1 K
	2	—			gone		
	3	14.5	+0.5		gone		
					15.0	not recorded previously	
	4	—			gone		

[1] In the case of a potential not being producible after the lesion it is listed in the table as "gone"; in the case of a potential appearing under circumstances where prior to the lesion no potential was seen the annotation "not recorded previously" has been made in the column for latency differences

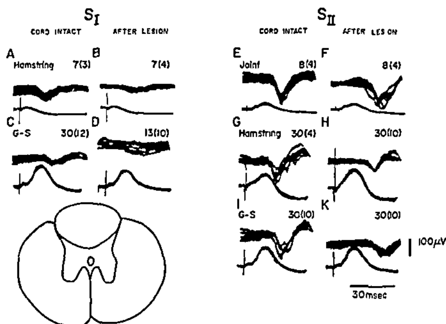


Fig 3 Potentials evoked in the hindlimb regions of the right somatosensory areas from left hindlimb nerves before and after transection of the left DLF Arrangement and symbols as in Fig 1

of the interosseous nerve In that experiment an interosseous evoked potential could be elicited in S_{II} at the usual strength i e less than twice the threshold of the nerve

The joint nerve Evoked potentials from the posterior joint nerve were recorded in both S_I and S_{II} in 5 expts out of 15 In 2 more expts they were found in either area but 60 per cent of the experiments gave negative results The latencies of these potentials varied considerably between different experiments in either area as evinced from the standard deviations given in tables The joint evoked potentials recorded in S_I (Fig 2, A and 4, C) were always of small amplitude whereas those recorded in S_{II} (Fig 2, G and 3, E) in some instances attained an amplitude comparable to that of the cutaneous evoked potentials The joint evoked potentials were never seen to develop simultaneously with the two first components of the afferent neurogram (cf Skoglund 1956) but appeared at stimulus intensities 2 5—8 times the threshold of the nerve

Muscle nerves Stimuli to either of the two muscle nerves gave rise to evoked potentials in both somatosensory areas (see Table I and II) These potentials, however, formed two groups with different characteristics One group labelled "early" in the tables was elicited at lower stimulus intensity and had shorter latency whereas the potentials labelled "late" in the tables were elicited at higher stimulation intensity and had longer latency

The "early" muscle evoked potentials recorded in S_I were in all instances elicited from the hamstring nerve (Fig 1, E, Fig 2, C and Fig 3, A) The threshold of these varied from 1 3—8 times the threshold of the nerve Thus in two experiments the hamstring "early" evoked potential was seen to develop before the group I spike had attained its maximum but in all the experiments it had started to develop before group III

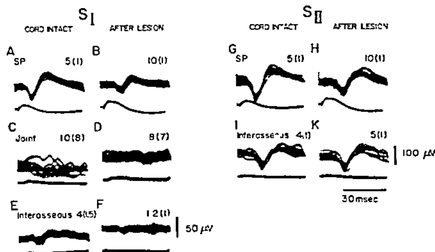


Fig 4 Potentials evoked in the hindlimb regions of the right somatosensory areas from left hindlimb nerves before and after transection of the left DLF The extent of the lesion was roughly similar to that shown in the drawing in Fig 3 Arrangement and symbols as in Fig 1

of the cutaneous evoked potentials after the lesion should be compared to the amplitude of the interosseous nerve potential which remained unaltered (records I and K

In the communications by Mark and Steiner (1958) and Norrsell and Voorhoeve (1962) it is stated that the latency of cortical potentials evoked from cutaneous nerves, whereas unaffected by lesions in the dorsal columns, is increased by about 2 msec by lesions in the DLF The differences in latency of the various evoked potentials before and after the lesions have been listed in separate columns in Table III and IV Whereas the latencies were almost unaltered following dorsal column transections they showed a marked increase for some of the groups after transections of the DLF Thus the cutaneous evoked potentials showed the expected increase in both areas The latencies of the muscle "late" evoked potentials and the potentials evoked from the posterior joint nerve increased as well (see Table III and IV) The latency of the potentials from the interosseous nerve did not vary significantly as a result of either lesion The data on the group of muscle "early" evoked potentials are somewhat incomplete In 2 expts where such potentials were recorded in S_{II} the potentials could not be evoked after lesions in the DLF The latency of one "early" potential recorded in S_{II} after a dorsal column transection had not changed nor had the latency of one recorded in S_I after a DLF lesion The only consistent finding with muscle "early" potentials was made with the group recorded in S_I after dorsal column transections where an average increase was found (Table III)

In 2 expts lesions of the spinal cord had been made at a low thoracic level prior to recording In the experiment of Fig 5 the whole of the spinal cord had been transected except the dorsal columns (see drawing) In the experiment of Fig 6 the lesion (see drawing) spared only the ipsilateral DLF After either lesion muscle evoked potentials of the "late" type could be recorded In addition cutaneous and interosseous evoked potentials were recorded with the dorsal columns intact and cutaneous and joint evoked potentials with the DLF intact

TABLE IV Latencies and differences from pre-lesion latencies of all evoked potentials recorded in both somatosensory areas after transection of the ipsilateral DLF[1]

Nerve	Experiment	Recorded in S_I Latency msec	Difference from prelesion value msec	Illustrated in figure	Recorded in S_{II} Latency msec	Difference from prelesion value msec	Illustrated in figure
Sural	5	9 5	+2 0		9 0	+1 0	
	6	—			*13 0*	*+5 0*	
	7	12 5	+2 5		11 5	+1 5	
	8	14 0	+3 5		20 0	+3 0	
	9	12 5	+2 5		18 0	+5 0	
Superficial peroneal	6	—			10 0	+1 5	
	7	11 5	−0 5		11 5	+1 5	
	8	14 0	+4 5		15 5	+3 0	
	9	*12 0*	*+1 5*	*4 B*	*13 0*	+2 5	4, H
Posterior joint	5	—			gone		
	7	25 0	+6 0		23 5	+6 5	3, F
	9	19 5	+1 0	4 D	gone		
Hamstring 'early group	7	13 5	+0 5	3 B	gone		
	9	gone					
late group	6	—			25 0	+8 5	
	7	—			22 0	not recorded previously	3 H
	8	26 0	not recorded previously		31 0	+4 5	
	9	—			27 0	+7 5	
Gastrocnemius soleus early' group	5	—			gone		
'late group	5	—			gone		
	6	—			gone		
	7	25 0	+6 0	3 D	29 0	+7 0	3 K
	8				37 0	+9 0	
	9	—			31 0	+9 0	
Interosseous	5	—			10 0	0	
	6				12 0	0	
	7	gone			9 0	−0 5	
	8	—			14 0	+1 0	
	9	13 0	0	4 F	13 0	−3 0	4 K

[1] Note see table III

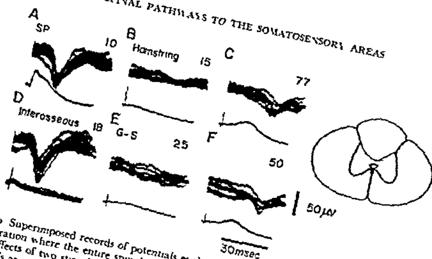


Fig 5 Superimposed records of potentials evoked in the contralateral S_{II} upper traces … preparation where the entire spinal cord except for the dorsal columns had been transect… The effects of two stimulation intensities are shown for the muscle nerves. Arrangement a… symbols as in Fig 6

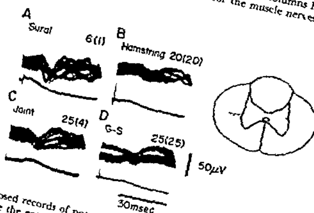


Fig 6 Superimposed records of potentials evoked in the contralateral S_{II} … preparation where the entire spinal cord except for the … The lower traces show records from the lumbar d… with the cortical records The stimulation intensitie… are given and the nerves indicated in conjunction … indicate the lowest intensity at which the cortica… was observed The … in parenthesis … refers to all records the voltage calibration to the upper traces The time calibration

Evoked activity in the somatosensory areas elicited via central spinal pathways

Though in some experiments no activity could be recorded in the somatosensory areas after both the dorsal columns and the DLF had been transected this was not always the case In Fig 7 is shown how a potential elicited in S_I by stimulating the SP nerve record A diminished in amplitude and increased in latency after transection of the DLF record B After transection of the dorsal columns an initially negative potential was recorded instead record C which was not altered by an extension of the lesion to include more than half the spinal cord record D and lower left hand drawing

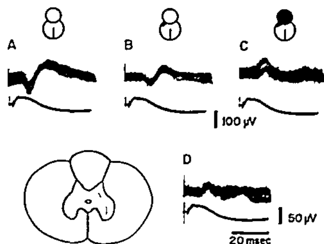


Fig. 7. Superimposed records of potentials, upper traces, evoked in the hindlimb region of the contralateral S_I from the SP nerve. In record A the spinal cord of the preparation was intact. Record B was taken after transection of the ipsilateral DLF. Record C was taken after the lesion had been extended to include the dorsal columns. The drawing in the left hand lower corner refers to record D taken when the whole of the dorsal columns, the ipsilateral spinal half and part of the contralateral ventral funiculus had been transected. Other arrangement and symbols as in Fig. 6.

The activity that could be evoked after transection of the dorsal columns and the DLF always consisted of initially negative potentials in both somatosensory areas. The amplitude was maximal at those points where the initially positive evoked potentials of maximal amplitude could be recorded in preparations with the dorsally located pathways intact.

A few experiments were performed to try to locate the pathway responsible for this transmission. The activity that could still be elicited after the two dorsally located ipsilateral pathways had been interrupted was unchanged unless the lesions were extended to include the ventral quadrant of the spinal cord contralateral to the stimulated nerves. With this part of the cord intact initially negative potentials could be elicited

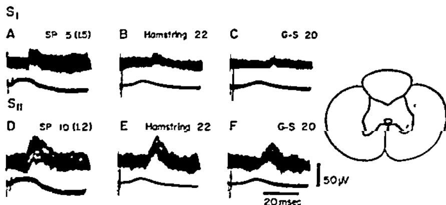


Fig. 8. Superimposed records of cortical potentials, upper traces, evoked in a preparation where all of the spinal cord except for the contralateral ventral quadrant and part of the ipsilateral ventral funiculus had been transected. The upper row depicts potentials elicited in the hindlimb region of the contralateral S_I and the lower row depicts potentials elicited in the hindlimb region of the contralateral S_{II}. Other arrangements and symbols as in Fig. 6.

in both somatosensory areas by stimulating cutaneous nerves at strengths less than twice the threshold and muscle nerves at strengths more than 10 times the threshold as shown in Fig 8

Discussion

The initially positive potentials elicited under Nembutal anesthesia in the cortical somatosensory areas from the contralateral hindlimb depend on two pathways in the spinal cord One is the well known dorsal column pathway and the other is one located in the ipsilateral DLF (Morin 1955) It has now been found that not only the effects from the cutaneous afferents (Mark and Steiner 1958, Norrsell and Voorhoeve 1962) but also those from high threshold muscle and joint afferents can be mediated equally well via either pathway

The cutaneous activation of S_I via Morin's pathway is transmitted in the spinal cord via the spinocervical tract (Norrsell and Voorhoeve 1962) This tract comprises two groups of axons activated from ipsilateral afferents which are located in the DLF and which terminate in the upper cervical segments (Lundberg 1964) One group is monosynaptically activated from small cutaneous fields and the other likewise monosynaptically from larger cutaneous fields and in addition, though probably polysynaptically, from high threshold muscle afferents (Lundberg and Oscarsson 1961) It is suggested that the non cutaneous effects that in the present investigation were shown to be transmitted in the DLF utilized the latter group of axons of the spinocervical tract This view is sustained by the work of Andersson (1962) and the same suggestion has previously been made on the basis of results obtained when recording from units of Morin's pathway at the lateral cervical nucleus relay (Gordon and Jukes 1963) There is so far no evidence in favour of the view (Morin *et al* 1963) that Morin s pathway is supplied at the lateral cervical nucleus by collaterals of the dorsal spinocerebellar tract (cf Lundberg 1964 for full discussion)

Cutaneous impulses are conducted to the somatosensory areas via the spinocervical tract more rapidly than via the dorsal columns (Mark and Steiner 1958 Norrsell and Voorhoeve 1962, Table IV) There is a corresponding but more pronounced difference between conduction in the two pathways from high threshold muscle and joint afferents (Table IV) This is probably due to the high conduction velocity in the spinocervical tract that will counterbalance the delay of extra synapses in Morin's pathway

It has been confirmed that initially positive potentials can be evoked in the somatosensory areas from group III muscle afferents (Mountcastle Covian and Harrison 1952) as well as from muscle afferents of larger diameter (McIntyre 1953, 1962a) The 'early' muscle potentials were evoked at intensities varying from 1 3 to 8 times threshold but although the responsible fibres were thus within the diameter range of muscle and tendon stretch receptor afferents (Eccles and Lundberg 1959) our findings (cf above) suggest that the afferents were of non proprioceptive origin The 'late'

the large muscle afferents that produce cortical evoked potentials is more obscure It has been suggested that they may serve Pacinian corpuscles or joint receptors (McIntyre 1962a) Since the effects from these afferents remained after transection of the dorsal columns these suggestions do not appear to be likely However, since some

afferents from the pressure sensitive receptors of muscles have been found to conduct at velocities of 70—90 m/sec (Paintal 1960) an alternative possibility could be that all hindlimb muscle afferents that project to the cortical somarosensory areas are activated by pressure stimuli That transmission of the muscle effects could occur via either of the two above mentioned pathways was best shown in 2 expts where initially positive potentials were evoked from muscle nerves in one case after transection of the entire spinal cord except for the dorsal columns (see Fig 5) and in the other after having spared only the ipsilateral DLF (see Fig 6) In addition the experiment of Fig 5 indicates that group III muscle afferents can ascend the length of the dorsal columns which has been disputed (cf Wall 1961, McIntyre 1962a)

The finding of McIntyre (1962b) that fibres from the hindlimb interosseous nerve activated at stimulus strengths just above the threshold project to S_{II} via the dorsal columns has been confirmed These afferents are presumably connected to vibration sensitive Pacinian corpuscles (McIntyre 1962b) In agreement with McIntyre it was found that S_I was activated from the interosseous nerve either at the same intensity that gave the large S_{II} potential or at higher intensities implying smaller diameter fibres as the source The high threshold activity remained after the dorsal columns had been *transected and could then also be fo nd in* S_{II} *Whether the high threshold fibres* of the interosseous nerve project to the somatosensory areas via the dorsal columns has not been investigated

Initially positive potentials were evoked from the joint nerve in both S_I and S_{II} from high threshold afferents following transection of either the dorsal columns or the DLF but not both indicating projection via pathways in either location No cortical effects were obtained at stimulation intensities that activated only the specific afferents i e those signalling joint position and movement On the other hand Skoglund (1956) found that potentials could be evoked in the contralateral S_I and S_{II} and the ipsilateral S_{II} from the specific afferents of the hindlimb medial and posterior joint nerves via the dorsal columns He found the effects to be more easily obtained with chloralose as compared to Nembutal anesthesia which since we have used Nembutal anesthesia throughout could explain our failure in this respect However the available evidence on the central projection of the hindlimb specific joint afferents is conflicting Thus there is evidence that they ascend the length of the dorsal columns (Gardner, Latimer and Stilwell 1949 Winter 1965) as well as evidence of unit activation from joints in S_I (Mountcastle 1957) Yet in contrast to Skoglund s (1956) finding the only unit activation from joints described in S_{II} has been attributed to non specific small di ameter fibres Andersson 1962 Carreras and Andersson 1963)

It has been confirmed that activity may be elicited in the somatosensory areas via contralateral spinal pathways (Gardner and Noer 1952 Gardner and Haddad 1953) The negative potentials encountered in the present investigation after a combined transection of the dorsal columns and the DLF (see Fig 7) may in spite of the polarity signify true projection since they appeared with topographical maxima in the somatosensory areas The site of the responsible ascending pathway is indicated by the finding

from the hindlimbs beginning low in the spinal cord and projecting to the ventrobasal complex of the thalamus as well as the posterior group of thalamic nuclei has previously been described in the cat (Whitlo k and Perl 1959

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References

ANDERSSON S A Projection of different spinal pathways to the second somatic sensory area in cat *Acta physiol scand* 1962 *56* Suppl 194

BUSCH H F N *An anatomical analysis of the white matter in the brain stem of the cat* Thesis Leiden 1961

CARRERAS M and S A ANDERSSON Functional properties of neurons of the anterior ectosylvian gyrus of the cat *J Neurophysiol* 1963 *26* 100—126

CATALANO J V and G LAMARCHE Central pathway for cutaneous impulses in the cat *Amer J Physiol* 1957 *189* 141—144

ECCLES R M and A LUNDBERG Synaptic actions in motoneurones by afferents which may evoke the flexion reflex. *Arch ital Biol* 1959 *97* 199—221

GARDNER E. and B HADDAD, Pathways to the cerebral cortex for afferent fibres from the hind leg of the cat *Amer J Physiol* 1953 *172* 475—487

GARDNER E F LATIMER and D STILLWELL, Central connections for afferent fibers from the knee joint of the cat *Amer J Physiol* 1949 *159* 195—198

GARDNER E and R NOER Projection of afferent fibres from muscles and joints to the cerebral cortex of the cat *Amer J Physiol* 1952 *168* 437—441

G [illegible]

L [illegible]

LUNDBERG A and O OSCARSSON Three ascending spinal pathways in the dorsal part of the lateral funiculus *Acta physiol scand* 1961 *51* 1—16

MARK R F and J STEINER Cortical projection of impulses in myelinated cutaneous afferent nerve fibres of the cat *J Physiol* (Lond) 1958 *142* 544—562

MCINTYRE A K Cortical projection of afferent impulses in muscle nerves *Proc Univ Otago med Sch* 1953 *31* 5—6

MCINTYRE A K Central projection of impulses from receptors activated by muscle stretch *Symposium on muscle receptors* ED BARKER Hong Kong Univ Press 1962a 19—29

MCINTYRE A K Cortical projection of impulses in the interosseous nerve of the cat s hind limb *J Physiol* (Lond) 1962b *163* 46—60

MORIN F A new spinal pathway for cutaneous impulses *Amer J Physiol* 1955 *183* 245—252

MORIN F S T KITAI H PORTNOY and C DEMIRJIAN Afferent projections to the lateral cervical nucleus a microelectrode study *Amer J Physiol* 1963 *204* 667—672

MOUNTCASTLE V B Modality and topographic properties of single neurons of cat s somatic sensory cortex *J Neurophysiol* 1957 *20* 408—434

MOUNTCASTLE V B M R COVIAN and C R HARRISON The central representation of some forms of deep sensibility *Res Publ Ass nerv ment Dis* 1952 *30* 339—370

NORRSELL U and P VOORHOEVE Tactile pathways from the hindlimb to the cerebral cortex in cat *Acta physiol scand* 1962 *54* 9—17

O [illegible]

C [illegible]

P [illegible] *Physiol* (Lond) 1960 *152* 250—270

SKOGLUND S Anatomical and physiological studies of knee joint innervation in the cat *Acta physiol scand* 1956 *36* Suppl 124

WALL, P D Two transmission systems for skin sensation *Sensory communication* Ed ROSENBLITH M I T Press 1961 475—495

WHITLOCK, D G and E R PERL Afferent projection through ventrolateral funiculi to thalamus of cat *J Neurophysiol* 1959 *22* 133—148

WINTER D L N gracilis of cat Functional organization and corticofugal effects. *J Neurophysiol* 1965 *28* 48—70

WOOLSEY C. N Pattern of sensory representation in the cerebral cortex *Fed Proc* 1947 *6* 437—441

Acta physiol. scand. 1966, 66, 34—48

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The Effect of Metabolic and Ion Transport Inhibitors on the Impulse Activity and Oxygen Uptake of an Isolated Crustacean Neurone

By

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Received 1 April 1965

Abstract

Giacobini E. *The effect of metabolic and ion transport inhibitors on the impulse activity and oxygen uptake of an isolated crustacean neurone.* Acta physiol scand 1966 *66* 34—48 — The effect of 19 metabolic and ion transport inhibitors acting at various enzymatic levels within several [illegible] suggests a coupling of respiration and impulse activity in the crustacean nerve cell The marked effect of the glycolytic inhibitors and of Na-malonate strongly suggests a link between glycolysis and Krebs cycle from one side and maintenance of impulse activity from the other This view is also supported by the effect of glucose restoring activity after the block caused by several inhibitors. Ouabain and digoxin inhibit both impulse activity and oxygen uptake In general respiration seems to be less sensitive to inhibitors than the impulse activity, indicating the presence of a safety factor for respiratory processes

A definite relationship between oxygen uptake and impulse activity has been established in isolated preparations of the slowly adapting cell of the stretch receptor organ of the crayfish (Giacobini Handelman and Terzuolo 1963, 1965, Giacobini 1964)

This finding poses the question of how the respiratory energy is elicited and utilized by the particular energy requiring mechanism (e.g. supplied in the form of energy rich compounds (adenosintriphosphate or arginine phosphate) or from individual steps or intermediates of respiration or glycolysis?

One of the simplest approaches which could give some indication of the selective pathways or steps involved, is to investigate the action of several metabolic inhibitors on the impulse activity and respiration, two parameters now readily measurable at the cellular level (Giacobini *et al* 1963

A preliminary report of the results reported in this paper was communicated at the XI Scand. Physiol. Congress in Copenhagen 1963

There are no reports in the literature of an extensive and systematic study of the action of metabolic inhibitors on isolated nerve cells. The investigation on isolated axons previously reported have, in general been limited to only a few compounds.

The aim of this investigation was to use a series of inhibitors acting at various enzymatic levels within each of several parts of the metabolic pathway and to compare their effect on the functioning of the nerve cell as judged by its impulse activity, and its metabolism, as expressed by oxygen uptake. Altogether 19 inhibitors were selected, acting on the respiratory chain, oxidative phosphorylation, citric acid cycle, glycolysis, -SH groups of enzymes, ion transport and finally on protein and ribonucleic acid synthesis (Toschi and Giacobini 1965). Each inhibitor was tried at various concentrations and pH values.

Materials and methods

et al. 1963) on an oscilloscope and audiomonitored. Frequency and total number of impulses were recorded by a digital counter (Venner Electronics TS A 3334).

Solutions of inhibitors were added in small volumes (1—10 μl) by means of a constriction

used

List of the inhibitors used with their source [1]

Intern

Results

Amytal at a concentration of 1 mM increased respiration whereas a concentration of 10 mM was completely inhibitory (Table I). The concentration needed to suppress impulse activity was somewhat higher than that which activated respiration. Glucose (1.3—2 mM) restored the electrical activity after the Amytal inhibition.

Na-cyanide at a concentration of 10 mM caused complete inhibition of respiration. However, a concentration of 1—2 mM gave 60 % inhibition without affecting impulse activity. This fact could indicate a safety factor of about 40 % for the correlation of impulse activity to oxygen uptake.

Antimycin A at a concentration of 0.025 mM completely blocked the impulse activity but a much higher concentration was needed to block respiratory activity (Table I).

[1] The generous gift of most of the listed inhibitors is gratefully acknowledged.

Table I. The effect of inhibitors of electron transfer on the electrical activity and the oxygen consumption of the slowly adapting neurone. The changes in spike height, impulse frequency and oxygen consumption are compared with the initial values before the addition of the inhibitor. The mean concentration of the inhibitor in at least 10 experiments is reported together with ± S.E.

Inhibitor	Conc. in mM	pH	Spike height	Impulse frequency	O_2 uptake × initial	Recovery
Amytal	1	7	7	—	3.5 ± 0.18	
Amytal	1.5 ± 0.04	7	3 → 1 ↘ 0	1 → 2 ↘ 0	—	Glucose 1.3—2 mM
Amytal	10	7	—	—	0	
Na-Cyanide	1	7	—	—	0.55 ± 0.08	
Na-Cyanide	2	7	—	—	0.65 ± 0.13	
Na-Cyanide	6.5 ± 1.8	7.5	2 → 1 ↘ 0	1 → 2 ↘ 0	—	
Na-Cyanide	10	8	—		0	
Antimycin A	0.025 0.001	7	1 → 1.5 ↘ 0	1 → 5 ↘ 0	—	Glucose no recovery
Antimycin A	0.2	7	—	—	0	
Rotenone	0.035 ± 0.032	6.8	1 → 0.5 ↘ 0	1 → ↘ 0		Glucose no recovery

A concentration as low as 3 × 10^{-3} mM rotenone (pH 6.8) prevented impulse initiation after stretch. Amytal, cyanide and rotenone progressively diminished the height of the action potential during the course of the experiment, while antimycin increased it slightly. Impulse frequency was augmented before the block by all 4 respiratory inhibitors, antimycin A and rotenone being the most active in this respect (Table I). It is interesting to note that in the case of rotenone the period (20—30 min) before total block was characterized by regular periodic bursts of activity.

2—4 dinitrophenol (2—4 DNP) at concentrations up to 0.2 mM (pH 6.8) did not affect the impulse activity (Table II), whereas the respiration of the resting preparation was increased at this concentration. At somewhat higher concentrations (0.3—0.4 mM) the cell no longer responded normally to stretch stimulation but a concentration of 0.75 mM was needed to completely block the impulse activity. The activity could be completely restored either by washing the preparation or by adding glucose (0.5—1.5 mM) or phosphoenolpyruvate (PEP) (2.5—5 mM). Adenosine triphosphate (ATP)

TABLE II The effect of uncoupling agents on the electrical activity and the oxygen consumption *of the slowly adapting neurone The changes in spike height impulse frequency* and oxygen consumption are compared with the initial values before the addition of the inhibitor The mean concentration of the inhibitor in at least 10 experiments is reported together with ±S E

Inhibitor	Conc in mM	pH	Spike height	Impulse frequency	O_2 uptake × initial	Recovery
2—4 Dinitrophenol	0 2	6 8	—	—	2 0±0 3	
2—4 Dinitrophenol	0 75±0 18	6 8	1 → 0 5 ↘ 0	1 → 2 ↘ 0	—	Glucose 0 3—1 3 mM PEP 2 5—5 0 mM
2—4 Dinitrophenol	2	7	—	—	3 7±0 9	
Na Arsenate	3	7	—	—	0 9±0 3	
Na Arsenate	21±2 9	7	1 → 0 75 ↘ 0	1 → 3 ↘ 0	—	
Na Arsenate	30	7	—	—	0 9±0 2	
Na Arsenate	60	7 5	—	—	3 7+0 4	
Dicoumarol	1	7	—	—	2 0±0 1	
Dicoumarol	0 011±0 065	7 8	1 → 0 65 ↘ 0	1 → 4 ↘ 0	—	Glucose 2 8—3 0 mM

and glucose 6 phosphate (G6P) showed no restoring effect (Table II) A concentration of 2 mM 2—4 DNP increased the oxygen uptake to almost 4 times the normal level

Sodium arsenate at concentration between 3 and 30 mM had a very slight inhibitory effect on the respiration while 60 mM produced an almost 4 fold stimulation (Table II) The concentration required for suppression of impulse activity was 21 mM Dicoumarol incre

impulse activity

tion The three u

inhibitory effects of varying degree on the action potential, 2-4-DNP being the most active Also the impulse frequency was increased by all 3 uncoupling agents before causing a block

Both the glycolytic inhibitors studied showed marked effects on the impulse activity and respiration (Table III) 2-deoxy-d glucose increased the respiration at 1 mM and suppressed it at 10 mM Sodium iodoacetate at concentrations between 2 and 10 mM inhibited the resting respiration by 40 % Concentration of 2 mM or above suppressed

TABLE III The effect of glycolytic and citric acid cycle inhibitors on the electrical activity and the oxygen consumption of the slowly adapting neurone The changes in spike height, impulse frequency and oxygen consumption are compared with the initial values before the addition of the inhibitor The mean concentration of the inhi bitor in at least 10 experiments is reported together with ± S E

Inhibitor	Conc in mM	pH	Spike height	Impulse frequency	O_2 uptake × initial	Recovery
2 Deoxy d Glucose	1	7	—	—	1 6±0 57	
2 Deoxy-d Glucose	2 0±0 26	7	1 → 0 5 → 0	1 → 5 → 0	—	Glucose 2 mM
2 Deoxy-d Glucose	10	7	—	—	0	
Na Iodoacetate	2	7	—	—	0 6±0 18	
Na Iodoacetate	5 0±0 45	7	1 → 0 75 → 0	1 → 12 → 0	—	
Na Iodoacetate	10	7			0 6+0 11	
Na Malonate	0 2+0 009	7	1 → 0 75 → 0	1 → 7 → 0		Glucose 3 mM

the effect of the stretch stimulation The spike height was reduced to 0 5 in the course of the experiment with 2 deoxy d glucose and to 0 75 of the initial with sodium iodoacetate The impulse frequency was markedly increased before block by both inhibitors 2 mM glucose restored the impulse activity after 2 deoxy d glucose

Na malonate in concentrations of 0 2 mM first inhibited the spike height by 25 % and increased the impulse frequency sevenfold and then finally blocked the impulse activity Glucose 3 mM could completely restore the impulse activity in the presence of the inhibitor (Table III)

Using sodium azide the impulse activity was not affected until a concentration of 5 mM (pH 7—7 5) was achieved and at 25 mM the inhibition was complete (Table IV) It should be noted that a 10 mM concentration increased respiration by a factor of 1 7 whereas a 20 mM concentration decreased it to 0 75 of the control

The other inhibitors of oxidative phosphorylation oligomycin and atractylate also produced a marked stimulation of respiration in the resting neurone Oligomycin suppressed the impulse activity at 2×10^{-3} mM and suppressed the respiration at 3×10^{-3} mM Glucose 1 3—5 mM restored impulse activity inhibited by oligomycin

Two inhibitors of oxidative phosphorylation atractylate and oligomycin had a more marked effect on the spike height sodium azide having very little effect Conversely

TABLE IV. The effect of inhibitors of oxidative phosphorylation on the electrical activity and the oxygen consumption of the slowly adapting neurone. The changes in spike height, impulse frequency and oxygen consumption are compared with the initial values before the addition of the inhibitor. The mean concentration of the inhibitor in at least 10 experiments is reported together with ± S.E.

Inhibitor	Conc. in mM	*pH*	Spike *height*	Impulse *frequency*	O_2 uptake × *initial*	*Recovery*
Na Azide	10	7.5	—	—	1.7 ± 0.7	
Na Azide	20	7	—	—	0.75 ± 0.02	
Na Azide	25 ± 4	7.5	1 → 1 → 0	1 → 5 → 0	—	
Atractylate	5	7	—	—	2.5 ± 0.4	
Atractylate	7 ± 1	7	1 → 0.5 → 0	1 → 2 → 0	—	
Atractylate	10	7	—	—	1.7 ± 0.4	
Oligomycin	1.5 (μM)	7	—	—	3.0 ± 0.3	
Oligomycin	1.8 (μM)	7		—	1.3 ± 0.02	
Oligomycin	2.1 ± 0.8 (μM)	7	1 → 0.5 → 0	1 → 4 → 0		Glucose 1.3–5.0 mM
Oligomycin	3.0 (μM)	7	—	—	0	

sodium azide had the greatest effect by progressively increasing the impulse frequency up to 5 times the initial before causing a block.

Ouabain and digoxin rapidly blocked impulse activity at concentrations of about 0.01–0.15 mM (Table V). Comparing the effect of ouabain in concentrations between 0.01 mM and 0.15 mM and controls in physiological solution it was found that the former preparations were able to fire on average about 1.1 × 10^3 impulses in total whereas the latter fired 360 × 10^3. If glucose was added (0.6 mM) the impulse activity recovered and the cell could fire an average of 1.3 × 10^4 impulses (see Table VI). ATP had no restoring effect. The respiration was more sensitive than the impulse activity to ouabain, a concentration of ouabain of 0.01 mM inhibiting the respiration of the resting cell by a factor between 80 and 30 %. It has been found that the effect on respiration was concentration dependent (Giacobini *et al.* 1965), low concentrations below 0.01 mM increasing respiration.

TABLE V. The effect of ouabain on the electrical activity of the slowly adapting neurone

Number of expts	Ouabain conc. in mM	Number of impulses	Duration of the expt. in min
15	*0.01—0.15*	*M = 10,900* (4,500 – 350,000)	*M = 146* (40 – 290)
10	Controls in physiol. solution	M = 360,000 (170,000 – 1,100,000)	M = 410 (240 – 720)
6	0.05—0.2 glucose[1] 0.6 mM	M = 1,300,000 (950,000 – 1,750,000)	M = 1,360 (1,100 – 1,700)

[1] Glucose was added after the block of the impulse activity due to ouabain. In 6 expt. ATP was added without any result. The mean M is reported together with the spread.

TABLE VI. The effect of mercaptide forming agents and ethanol on the electrical activity and the oxygen consumption of the slowly adapting neurone. The changes in spike height, impulse frequency and oxygen consumption are compared with the initial values before the addition of the inhibitor. The mean concentration of the inhibitor in at least 10 experiments is reported together with ±S.E.

p-Hydroxy Mercuribenzoate	0.1	7.5	—	—	0.95 ± 0.2	
p-Hydroxy Mercuribenzoate	0.25 ± 0.01	7.5	1 → 0.75 → 0	1 → 0.5 → 0	—	
p-Hydroxy Mercuribenzoate	1	7.5	—	—	0.80 ± 0.01	
p-Hydroxy Mercuribenzoate	2	7.5	—	—	0.60 ± 0.04	
Ethyl-Alcohol	1.75 ± 0.37	7	1 → 8 → 0	1 → 3 → 0	—	Glucose 1.5 – 10 mM short recovery
Ethyl Alcohol	21	7	—	—	2.5 ± 0.03	
Ethyl-Alcohol	54	7	—	—	4.1 ± 0.04	
Ethyl Alcohol	65	7	—	—	3.9 ± 0.03	

One of the most commonly used mercaptide reagent, p-hydroxymercuribenzoate (pH 7.5) was tried. The impulse activity was very sensitive to this compound and at a concentration as low as 0.25 mM it was completely abolished. An almost 10 times higher concentration was necessary to inhibit respiration by 40 % (Table V). Both

spike height and frequency were influenced during the experiments with 0.25 mM p-hydroxymercuribenzoate

Ethyl alcohol was tried at increasing concentrations from 21 to 65 mM with the constant effect of increasing the oxygen uptake of the cell up to fourfold the initial (Table V) At a much lower concentration than above (1 75 mM) the cell was no longer able to respond to stretch stimulation 1 5 mM glucose could restore the electrical activity of the cell for short periods of time only (5—15 min)

As demonstrated by *Toschi and Giacobini* (1965), *puromycin at concentrations* of 125—250 μg/ml and actinomycin at concentrations of 1—2 5 μg/ml did not have any effect either on the respiration or the electrical activity of the isolated neurone of the crayfish

Discussion

Inhibitors of electron transfer

Rotenone, believed to block electron transport at the flavoprotein level (Ernster, Dallner and Azzone 1963) was found to be the most effective inhibitor of impulse activity, acting at concentrations of 10^5 mM Antimycin A, acting between cytochrome b and c_1 in mitochondrial preparations (Chance 1952) also showed a pronounced inhibitory effect both on respiration and electrical activity It is possible that such a relatively high concentration of antimycin could exert some uncoupling effect in the upper part of the respiratory chain Furthermore an inhibitory effect on the oxidation of the Krebs cycle and on the succinooxidase system by antimycin A has been reported (Potter and Reif 1952) To our knowledge the effect on isolated axon or nerve cell preparations of these inhibitors had not been previously described Cyanide however has been extensively used in neurophysiological investigations on single nerve fiber preparations (Schoepfle and Bloom 1959, Schoepfle 1963, Brady, Spyropoulus and Tasaki 1958, Keynes 1959, Hodgkin and Keynes 1955, Caldwell 1955)

Schoepfle and Bloom (1959) and Schoepfle (1963) showed that exposure of frog *single nerve fibers to cyanide (2—10 mM) resulted in a decline in spike height and* Schoepfle (1963) pointed out that cyanide should block a chain of reactions at a link remote from the step directly concerned with the maintenance of sodium conductance A concentration as high as 400 mM had no immediate effect upon the action potential when microinjected intraaxonally in the giant axon (Brady, Spyropoulus and Tasaki 1958) According to Keynes (1959) 2 mM cyanide at pH below 7 had no marked effect on the ability of the isolated squid axon to conduct electrical impulses even 5 hrs and more after exposure to the inhibitor

The above observations suggested that the immediate source of energy for the production of a nerve impulse is largely independent of metabolic mechanisms In our experiments at pH 7 5 a concentration of 6 5 mM cyanide was needed to suppress impulse activity Amobarbital, which is believed to inhibit both *electron transport and energy* transfer (Ernster *et al* 1963) had a stimulatory effect on respiration at low concentrations (1 mM) and a marked inhibitory effect at higher concentrations (10 mM thus differing from its action on cell fractions and tissue slices. The stimulatory effect could be due to uncoupling of oxidative phosphorylation as described by Brody and Bain (1951) It can be concluded that the effect of antimycin A and rotenone which have been found to be firmly bound to mitochondria and to act on mitochondrial respiration see Ernster and Lee 1964) supports a correlation between electron transport mechanism

TABLE VII Concentrations of 19 metabolic inhibitors suppressing impulse activity and inhibiting or activating oxygen uptake together with the concentration of substrates restoring impulse activity

Type of inhibitor	Inhibitor	Conc of inhibit suppressing imp *activity in* mM	Conc of substr restoring imp *activity in* mM	Conc inhibiting or activating *O_2 uptake in* mM	*% inhibition* of O_2 uptake
Inhibitors of electron transfer	Amytal	1 3±0 04	Glucose 1 3 2	10	100
	Na-cyanide	6 5±1 8	—	10	100
	Antimycin A	0 025±0 001	Glucose no effect	0 2	100
	Rotenone	0 035±0 002 (μM)	Glucose no effect	—	—
Uncoupling agents	2—4 DNP	0 75±0 018	Glucose 0 3—1 3 PEP 2 5—5 ATP G6P no effect	0 2—2	Activation
	Na arsenate	21 0±2 9	—	30	10
	Dicoumarol	0 01±0 006	Glucose 2 8 3	1	Activation
Glycolytic inhibitors	2-deoxy d glucose	2 0±0 26	Glucose 2	10	100
	Na iodoacetate	5 0±0 45	—	2—10	40
Citric acid cycle inhibitors	Na malonate	0 02±0 009	Glucose 3		—
Inhibitors of oxidative phosphorylation	Na azide	25±4	—	20	25
	Oligomycin	2 1±0 8 (μM)	Glucose 1 3 5	3 (μM)	100
	Atractylate	7+1	—	5 10	Activation
Inhibitors of ion transport	Ouabain	0 12+0 008	Glucose 0 6 ATP no eff	0 01	20 70
	Digoxin	0 09+0 002	Glucose 50 ATP no eff	0 01	30—70
Mercaptide forming agents	p hydroxy mercuri benzoate	0 25±0 01	—	2	40
Alcohols	Ethyl alc	1 75+0 37	Glucose 1 5 (slight eff)	21—65	Activation
Inhibitors of protein and RNA synthesis	Puromycin	125 250 μg ml no effect	—	125—250 μg/ml	No effect
	Actinomycin D	1 25 μg ml no effect	—	1—2 5 μg/ml	No effect

and impulse activity The effect of these inhibitors on the respiration of the isolated neurone suggests that only a small part of it may be connected to the impulse generating mechanism as concentration of inhibitor blocking impulse activity in general only partially inhibits respiration (Table VII)

b) *Uncoupling agents*

According to Hodgkin and Keynes (1955) 0.2 mM DNP had no effect on the conduction of isolated squid axons They reported that although there were large changes in the resting sodium efflux, the sodium movements during the spike appeared to be unaltered when DNP was applied Shanes (1951, b) working with isolated squid axons had previously demonstrated failure of conduction after DNP Straub (1956) found only slight changes in resting and action potentials of frog nerve fibres after 30 min treatment with 0 2 mM DNP Dettbarn and Stampfli (1957), using the sucrose gap technique on de-sheated nerve fibres of frogs and rats found that DNP at pH 6—7 8 reduced the membrane potential and depolarization In our experiments the concentration needed to block impulse activity was 3 times higher than that used by the above authors We confirmed the results of Hodgkin and Keynes by showing that 0 2 mM DNP (pH 6 8) did not exert any effect on the impulse activity The inhibitory effect of DNP could be restored very quickly by glucose or PEP whereas ATP and G6P had no effect As reported in experiments on particulate material, DNP increased the resting respiration several fold (Table VII)

Na arsenate a competitive inhibitor of phosphate, and dicoumarol believed to cause breakdown of high energy intermediates, affected the impulse activity at concentrations of 21 mM and 0 01 mM respectively Siekewitz *et al* (1958) demonstrated that 20 mM Na arsenate gave a 50 % inhibition of ATPase activity in rat liver mitochondria The slight effect of Na arsenate is in agreement with the well known properties of this compound as a weak uncoupler

c) *Glycolytic inhibitors*

The depression, by iodoacetate of the membrane potential and conduction is indirect evidence for the implication of glycolysis in the electrical processes of the nerve (Ronzoni 1931 Lorente de No 1947) Chang and Gerard (1933) observed that iodoacetic acid (IAA) depressed the oxygen uptake and at a concentration of 5 mM one third of the creatine phosphate content of the nerve was lost In their early work Shanes and Brown (1942) found that soaking sciatic nerves of frog in IAA reduced the resting potential this effect of IAA being counteracted by lactate and pyruvate Later, Shanes (1951 a) showed that addition of glucose could delay the anoxic change of ionic content

Electrical stimulation of IAA (1 5—10 mM) treated slices of electric organ (Maitra *et al* 1964) gave high levels of fructose 1 6-diphosphate and triose phosphate In the presence of 5 mM Na IAA our crustacean preparation was no longer able to respond to physiological stimulation It is interesting to note that a twofold higher concentration of Na IAA inhibited only 40 % of the total oxygen resting consumption (Table VII)

d) *Citric acid cycle intermediates*

In studies connected with CAC in peripheral nerves two inhibitors methyl fluoroacetate and sodium fluoroacetate have been commonly used According to Bovarsky *et al* (1949) methyl fluoroacetate 1—5 mM) inhibited the rate of oxygen uptake and

ultimately blocked conduction of impulses Both succinate and fumarate prevented this action if added before the inhibitor From these studies it was suggested that the tricarboxylic acid cycle utilizing pyruvate was probably active in the peripheral nerve The literature reveals no comparable study on isolated neurone preparations

In our experiments sodium malonate gave suppression of the impulse activity at 0 02 mM a concentration lower than that required to affect respiration of cortex slices (Quastel 1958 Wallgren 1960) or mitochondrial preparations (Brierley *et al* 1963

e) Inhibitors of oxidative phosphorylation

In our preparation azide at concentrations above 5 mM affected the impulse activity but a complete block was not obtained until a concentration of 25 mM was reached Hodgkin and Keynes (1955) and Hurlbut (1958—1963) demonstrated marked reduction of Na efflux with similar concentrations (3—5 mM) of Na azide in giant axons and frog nerves respectively At a concentration of 5 mM Siekewitz *et al* (1958) found an almost 90 % inhibition of the ATPase activity in rat liver mitochondria

It is interesting to note that at concentrations of 10 mM at pH 7 5 the oxygen uptake was increased but at that concentration inhibiting impulse activity (25 mM) respiration was also inhibited (Table IV) This is in agreement with the established fact that this inhibitor is less active in vivo than in vitro and stimulates respiration at pH 7 or above (see Hewitt and Nicholas in Hochster and Quastel 1963) Doty and Gerard 1950 showed that this compound can suppress the increase in oxygen consumption without affecting the ability of the axon to conduct impulses This finding is obviously not in agreement with our results

Oligomycin which prevents the formation of an energy rich intermediate of oxidative phosphorylation Lardy Johnson and McMurray 1958) affected respiration at about the same concentration as that acting on mitochondrial preparations (Lardy *et al* 1958 Wadkins and Lehninger 1963 Atractylate which is believed to have an action different from oligomycin and to interfere with the terminal phase of energy transfer reactions Santi 1964 required a rather high concentration to inhibit impulse activity and at this level exhibited an excitatory action on respiration Table VII)

f Inhibitors of ion transport

In previous papers Giacobini 1964 Giacobini *et al* 1965 the effect of ouabain on the oxygen uptake has been examined and a biphasic effect has been described, viz low concentrations 10 ⁻ — 10 ⁻ M being stimulatory whilst higher concentrations (above 10 ⁻ M inhibit endogenous respiration. As summarized in Table V some effect on the impulse activity was noticeable even at a concentration of 1 × 10 ⁻ M However to suppress the impulse activity completely a concentration of 10 ⁻ M was necessary At this concentration in the presence of 5 5 mM K ATPase activity should also be suppressed Palmer 1964 Respiration however was inhibited by a tenfold lower concentration.

g) Mercaptide forming agents

P hydroxy mercuribenzoate PHMB the most commonly used of the mercaptide forming agents, was shown to act on different sulphydryl enzymes at much higher concentrations than those acting on the impulse activity of the crustacean neurone (for ref see Madsen in Hochster and Quastel 1963 This could suggest that the inhibi

tory mechanism operating on this cell is of a different nature At this concentration (0 2 mM) ATPase may be totally inhibited as was demonstrated by Siekewitz *et al* (1958) for rat liver mitochondria The fact that this inhibitor is known to act on glycer aldehyde-3 phosphate dehydrogenase and phosphorylase (for ref see Madsen in

h) *Ethyl alcohol*

The crustacean nerve cell was found to be more sensitive to ethyl alcohol than nerve tissue slices increasing the oxygen consumption two—fourfold In tissue slices 40—80 mM concentrations increased the oxygen consumption by only 10 % (Wallgren and Kulonen 1960 Quastel 1959)

High concentrations of ethyl alcohol were found to produce depolarization of the frog nerve membrane (Bishop 1932) and an inhibitory effect of the frog skin potential with impairment of the Na transport at concentrations of 40—300 mM has been reported (Israel and Kalant 1963)

The effect of alcohols on Na and K permeability of nerve fibres has recently been confirmed by Armstrong and Binstock (1963—1964) in the squid giant axon and by Moore Ulbricht and Takata (1964) on the same material with a voltage clamp technique

i) *Inhibitors of protein and RNA synthesis*

The action of these inhibitors on the electrical activity and the respiration of the crustacean nerve cell has been the subject of a detailed report (Toschi and Giacobini 1965) Here it is pertinent to mention only that in acute experiments lasting 9—12 hours the complete inhibition of protein and RNA synthesis did not affect either the respiration or the impulse activity of the cell These results agree with those reported by Grampp and Edstrom (1963 on the lobster stretch receptor cell where generation and conduction of about 10 10^4 spikes during several hours did not alter the total amount of RNA

General conclusions

Our study of the effect of inhibitors on a living cell preparation suffers from some limitations Firstly the well defined action of these inhibitors in vitro (e g on mitochondrial preparations) may not resemble that on single isolated cells where there is a more complex metabolic system This discrepancy has been described on several occasions both for respiratory inhibitors and inhibitors of oxidative phosphorylation (for a complete review of this argument see Hewitt and Nicholas in Hochster and Quastel 1963 p 414 vol II) It is evident that these circumstances may affect the interpretation of our data for example when we observe that azide or cyanide does not inhibit oxygen uptake as much as might be expected from experiments in vitro Conversely it is hoped that our study of the action of metabolic inhibitors on isolated cells may give more direct information about the physiological mechanisms acting in vivo

There are other factors to be considered when interpreting our results It is essential to know whether each inhibitor reaches the sensitive point in the cell It is known that pH influences the rate of penetration of certain inhibitors both respiratory inhibitors

and uncoupling agents seem to be examples (For a discussion see Hewitt and Nicholas in Hochster and Quastel, 1963, p 414—420, vol II) A further point to be considered is the specificity of the inhibitor Some of the inhibitors which we have been using have been assumed to be absolutely specific for a certain system, e g for the electron transfer chain or for oxidative phosphorylation However, as it has been pointed out by Green *et al* (1963), all the classical inhibitors of oxidative phosphorylation and uncoupling agent can also inhibit other enzyme systems

Bearing in mind these limitations it is possible to make only tentative suggestions about the selective metabolic pathways involved The effect of the respiratory inhibitors especially rotenone and antimycin A, as previously commented suggests a coupling of respiration and impulse activity in the crustacean nerve cell This is also in agreement with the reported presence and activity of respiratory enzymes and cytochromes (Munro 1955) in crustacea, and with the findings of Terzuolo *et al* (1961) and Bonewell and Giacobini, that the activity of oxidative enzymes is increased during impulse activity It has also been suggested that glycolysis, tricarboxylic acid cycle and the electron transfer system are the major metabolic pathways for crustacea (for a complete review see Volkekamp and Waterman in Waterman 1960, vol I chapter 2, Puyear, Wang and Pritchard 1964)

It can be seen from the results summarized in Table VII, that respiration in general is less sensitive to inhibitors than is the impulse activity This could indicate a safety mechanism for respiratory processes, probably needed for other mechanisms in addition to impulse propagation The only exception to this is the effect of the inhibitors of ion transport but it should be noted that these compounds not only affect respiration but are also powerful inhibitors of ATPase activity

The marked effect of the glycolytic inhibitors and of Na malonate strongly suggests a link between glycolysis and Krebs cycle from one side and maintenance of impulse activity from the other This view is also supported by the effect of glucose restoring activity after the blocks caused by several inhibitors as reported in Table VII

If we are to decide whether the inhibitory action is related to active transport processes it will be necessary to determine Na and K in cells treated with different inhibitors This is in progress by means of a modified microflamephotometer which makes possible the simultaneous measurement of K Na and Ca (Giacobini 1965) We must recognize that the final answer to our questions cannot be given by an inhibitory study alone but must be complemented by the determination of actual intermediates involved in the specific metabolic systems This can possibly be achieved by following the level of each substrate in different conditions at rest and during physiological stimulation see Grasso and Giacobini 1965 this journal)

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References

ARMSTRONG C M and L BINSTOCK The effect of several alcohols on the properties of the squid giant axon *J gen Physiol* 1964 *48* 265—277

BINSTOCK L and C M ARMSTRONG Effect of a series of alcohols on voltage clamped squid axons *Abstr bioph Soc* 1963 *7* WC 3

BISHOP G H The relation of nerve polarization to monophasicity of its response *J cell comp Physiol* 1932 *1* 371—386

BONNEWELL G and E GIACOBINI To be publ

BOYARSKY, L L A D ROSENBLATT S POSTEL and R W GERARD Action of methyl fluoroacetate on respiration and potential of nerve *Amer J Physiol* 1949 *157* 291—316

BRADY R O C SPYROPOULOS and I TASAKI Intraaxonal injection of biologically active materials *Amer J Physiol* 1958 *194* 207—213

BRIERLEY G E MURER E BACHMANN and D E GREEN Studies on ion transport *J biol Chem* 1963 *238* 3482—3489

BRODY T M and J A BAIN Effect of barbiturates on oxidative phosphorylation *Proc Soc exp Biol* (N Y) 1951 *77* 50—53

CALDWELL P C The effect of certain metabolic inhibitors on the phosphate esters of the squid giant axon *J Physiol* (Lond) 1956 *132* 35

CHANCE, B Spectra and reaction kinetics of respiration pigments of homogenized and intact cells *Nature* (Lond) 1952 *169* 215—221

CHANG T H and R W GERARD Studies on nerve metabolism *Amer J Physiol* 1933 *104* 291—297

DETTBARN W D and R STAMPFLI Die Wirkung von 2—4 Dinitrophenol auf das Membranpotential der markhaltigen Nervenfaser *Helv physiol pharmacol Acta* 1957 *15* 26—37

D

E

E

C

C

GIACOBINI E 1965 To be publ

GIACOBINI E E HANDELMAN and C TERZUOLO An isolated neurone preparation for studies of metabolic events at rest and during impulse activity *Science* 1963 *140* 74—75

GIACOBINI E F HANDELMAN and C TERZUOLO Changes in O_2 uptake by a single neurone under different experimental conditions 1965 In press

GIACOBINI E and A GRASSO Variations of glycolytic intermediates phosphate compounds and pyridine nucleotides after prolonged stimulation in an isolated crustacean neurone *Acta physiol scand* 1966 *66* 49—57

GIACOBINI E and MARCHISIO P C The action of tricarboxylic acid cycle intermediates and glutamate on the impulse activity and respiration of the crayfish stretch receptor neurone *Acta physiol scand* 1966 *66* 58—66

GRAMPP W and J E EDSTROM The effect of nervous activity on ribonucleic acid of the crustacean receptor neurone *J Neurochem* 1963 *10* 725—731

GREEN D E R D BEYER M HANSEN A L SMITH and G WEBSTER Coupling factors and the mechanism of oxidative phosphorylation *Fed proc* 1963 *22* 1460 1468

HOCHSTER R M and J H QUASTEL *Metabolic inhibitors* Acad Press (London and New York) 1963

HODGKIN A L and R D KEYNES Active transport of cations in giant axons from Sepia and Loligo *J Physiol* (Lond) 1955 *128* 28—37

H

H

I

KEYNES R D Metabolism and activity in giant axons *Biochemistry of the central nervous system* Edited by Brucke Pergamon Press 1959 *3* 18—25
LARDY, H A D JOHNSON and W C McMURRAY, Antibiotics as tools for metabolic studies. *Arch Biochem* 1958 *78* 587—597
[illegible]
conductances of the squid giant axon *J gen Physiol* 1964 *48* 279—295
MUNRO F H The effect of oxygen on the concentration of haemin in invertebrates *Proc roy Soc B* 1955 *143* 203—214
PALMER R Pers comm 1964
POTTER V and A E REIF Inhibition of an electron transport component by antimycin A *J biol Chem* 1952 *194* 287—297
PUYEAR, R L C H WANG and A W PRITCHARD Catabolic pathways of carbohydrate on the intermolt crayfish Pacifastacus Leniusculus *Comp Biochem Physiol* 1965 *14* 145—153
QUASTEL J H Enzymatic mechanism of the brain and the effects of some neurotropic agents *Biochemistry of the central nervous system* Edited by Brucke Pergamon Press 1959 *3* 90—114
RONZONI E The source of energy of nerve activity *J biol Chem* 1931 *92* 3—4
SANTI R Pharmacological properties and mechanism of action of atractyloside *J Pharm Pharmacol* 1964 *16* 437—438
SCHOEPFLE G M and F E BLOOM Effects of cyanide and dinitrophenol on membrane properties of single nerve fibres *Amer J Physiol* 1959 *197* 1131—1135
SCHOEPFLE G M Effects of cyanide at different concentrations on single nerve fibres *Amer J Physiol* 1963 *204* 77—80
SHANES A M a) Factors in nerve functioning *Fed Proc* 1951 *10* 611—621
SHANES A M b) Potassium movements in relation to nerve activity *J gen Physiol* 1951 *34* 795—807
SHANES A M and E S BROWN The effect of metabolic inhibitors on the resting potential of frog nerve *J cell comp Physiol* 1942 *19* 1—13
SILKEWITZ P H LOW L LRNSTER and O LINDBERG On a possible mechanism of the adenosinotriphosphatase of liver mitochondria *Bioch Bioph Acta* 1958 *29* 378—391
STRAUB R Die Wirkungen von Veratridin und Jonen auf das Ruhepotential markhaltiger Nervenfasern des Frosches *Helv physiol pharmacol Acta* 1956 *14* 1—28
TERZUOLO C G BONEWELL E GIACOBINI T HANDELMAN and S LIN Metabolic studies in a single isolated nerve cell *Fed Proc Abstr* 1964 *23* part I *130* 73
TOSCHI G and E GIACOBINI Life Sciences 1965 *4* 1831—1834
WADKINS C L and A L LEHNINGER Role of ATP ADP exchange reaction in oxidative phosphorylation *Fed Proc* 1963 *22* 1092—1096
WATERMAN T H *The physiology of crustacea* Acad Press (London and New York) 1960
WALLGREN H Comparison of the effect of ethanol and malonate on the respiration of rat brain cortex slices *Acta physiol scand* 1960 *49* 216—223
WALLGREN H and F KULONEN Effect of ethanol on respiration of rat brain cortex slices *Biochem J* 1960 *75* 150

Acta physiol scand 1966 66 49—57

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Variations of Glycolytic Intermediates, Phosphate Compounds and Pyridine Nucleotides after Prolonged Stimulation of an Isolated Crustacean Neurone

By

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Abstract

Giacobini E and A Grasso *Variations of glycolytic intermediates phosphate compounds and pyridine*

decrease during prolonged stimulation while ADP and Pi showed opposite patterns After stimulation the ratio TPN/TPNH fell by more than 50 per cent. This was due to a decrease of TPN Similarly the DPN/DPNH ratio fell by 50 per cent but in this case it was due to a decrease of DPN and a simultaneous increase of DPNH The results strongly suggest the involvement of glycolysis in the mechanism maintaining impulse activity in this neurone Impulse activity in the crustacean nerve cell is linked to an energy requiring system This energy is furnished by ATP splitting and glycolysis directly or through the arginine phosphate step supporting the ATP resynthesis

Probably the earliest direct evidence for an involvment of glycolytic processes in isolated nerves was produced by Gerard and Meyerhof (1927) who showed the utilization of glucose and the formation of lactate in anoxic frog nerves This was later confirmed in similar studies with rabbit and bullfrog nerve (Gerard 1932) Schmitt and Cori (1933) demonstrated the oxidative removal of the anoxically formed lactate However there was no evidence from these studies that the conduction of impulses increased the rate of production of lactate

The following abbreviations were used in the text G6P = glucose-6-phosphate Pi = inorganic phosphate [illegible]
adenos ne [illegible]
triphosphc [illegible]
d phosphate [illegible]
pyruvate [illegible]

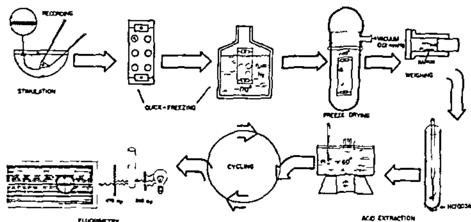


Fig 1 Schematic description of the analytical procedure (see text)

Sodium iodoacetate, a glycolytic inhibitor was later shown to have a depressor effect upon the membrane potential (Shanes and Brown 1942 and Shanes 1949) and conduction (Ronzoni 1931, Lorente de No 1947) Thus glycolysis was linked to the maintenance of potentials in the nerve

Estimation of glycogen, "free carbohydrate" and lactic acid in crustacean nerve tissue was performed firstly by Holmes (1929), who demonstrated that the peripheral nerves and ganglia of these invertebrates are extremely rich in glycogen and that, in anaerobic conditions, this is hydrolysed to form lactic acid In the presence of oxygen there was less breakdown of glycogen and no lactic acid was formed

Our previous study (Giacobini 1965), with metabolic inhibitors indicated glycolysis as a probable source of substrates for oxidative processes linked to impulse activity in the slowly adapting receptor cell of crayfish For this reason we decided to investigate the level of several glycolytic intermediates with a newly described biochemical technique which allows the estimation of these substrates at the cellular level (Lowry *et al* 1964) Phosphate compounds and pyridine nucleotides were also determined in the isolated cell preparation, both at rest and after prolonged physiological stimulation, using the same method

Methods

The slowly adapting stretch receptor organs used in this investigation were dissected from abdominal segments of crayfish (*Orconectes virilis*) Only young specimens were used, measuring about 5—8 cm in total body length The animals were collected during spring and autumn [illegible] were performed at 17—18° C

Preparation of cells

A single receptor cell (slowly adapting cell) was isolated and suspended in a small petri dish containing 2 ml van Harreveld solution The muscle bundle was mounted on a mechanical

TABLE I Analytical conditions. The conditions reported in the table are approximately those described by Lowry et al. (1964). For glycogen and inorganic phosphate a direct method with 1 ml volume was used. For glucose, G6P, lactate, ATP and ADP the indirect procedure of the cycling system was used. The cycling conditions for both the pyridine nucleotides were those reported by Lowry (1963). The measurement of pyridine nucleotide was done by means of the cycling procedure. The experimental conditions were those reported by Lowry et al. (1961). All reagents contained 0.01 % BSA.

Substance	Buffer[1]	Enzyme	Other additions	Incubation time in min	Reaction volumes	No of cells
Glucose	Tris 100 mM pH 7.5	HK 2 γ/ml yeast G6PDH 0.5 γ/ml yeast	ATP 0.1 mM TPN 0.03 mM $MgCl_2$ 5 mM	20	2 µl HCl 0.03 N 5 µl glucose reagent 5 µl NaOH 0.02 N 100 µl cycling (TPNH) system	58
G6P	Tris 100 mM pH 8.0	G6PDH 0.25 γ/ml yeast	TPN 0.03 mM	10	2 µl HCl 0.03 N 5 µl G6P reagent 5 µl NaOH 0.02 N 100 µl cycling (TPNH) system	18
Lactate	Carbonate 200 mM pH 9.7	LDH 5 γ/ml pig heart	DPN 0.5 mM Hydrazine Hydrate 50 mM	15	1 µl HCl 0.03 N 5 µl lactate reagent 5 µl NaOH 0.02 N 50 µl cycling (DPNH) system	26
Glycogen	Imidazole 20 mM pH 7.0	PHRL-a 30 γ/ml rabbit muscle PGM 3 γ/ml rabbit muscle G6PDH 0.5 γ/ml yeast	$MgCl_2$ 2 mM TPN 0.04 mM AMP 0.2 mM Pi 10 mM	20	3 µl HCl 0.03 N 10 µl glycogen reagent 10 µl NaOH 0.02 N 200 µl NaOH 7 N H_2O_2 0.3 % ad 1 000 µl H_2O	82
Pi	Imidazole 20 mM pH 7.0	PHRL-a 30 γ/ml rabbit muscle PGM 3 γ/ml rabbit muscle G6PDH 0.5 γ/ml yeast	$MgCl_2$ 2 mM TPN 0.04 mM AMP 0.2 mM Glycogen 5 mM	20	5 µl HCl 0.03 N 15 µl Pi reagent 15 µl NaOH 0.02 N 200 µl NaOH 7 N H_2O_2 0.3 % ad 1 000 µl H_2O	20

Table I. Cont

Substance	Buffer[1]	Enzyme	Other additions	Incubation time in min	Reaction volumes	No of cells
ATP	Tris 100 mM pH 7 5	HK 1 γ/ml yeast G6PDH 0 25 γ/ml yeast	$MgCl_2$ 2 mM TPN 0 03 mM Glucose 1 mM	10	1 μl NaOH 0 1 N 1 μl HCl 0 1 N 5 μl ATP reagent 5 μl NaOH 2 N 100 μl cycling (TPNH) system	22
ADP[2]	Phosphate 50 mM pH 7 0	PK 0 5 γ/ml rabbit muscle LDH 0 25 γ/ml pig heart	$MgCl_2$ 2 mM PEP 0 5 mM ATP 0 5 mM DPNH 0 15 mM	10	2 μl HCl 0 03 N 5 μl ADP *reagent* 5 μl HCl 0 2 N 100 μl cycling DPN reagent	18

[1] The organic buffers have been charcoal treated in order to decrease the natural fluorescence

[2] This method will also give values for GDP, IDP, UDP (Strominger 1955)

HK = hexokinase G6PDH = glucose 6-phosphate dehydrogenase, LDH = lactic dehydrogenase, PHRL phosphorylase BSA = bovine serum albumin.

stretcher so that different degrees of stretch could be applied to the muscle Impulse activity was recorded by means of a 30 μ thin wire electrode and displayed on an oscilloscope and audiomonitored (Fig 1) The total number of impulses as well as the impulse frequency, was recorded on a digital counter (Venner Electronics TS A3331) A thin layer of mineral oil covered the surface of the solution to prevent evaporation (Fig 1) After a number of impulses the cell was trimmed to the standard dimensions whilst in the mechanical stretcher, and quickly transferred by means of a thin glass fiber to a precooled Lowry-holder (Lowry 1953) (Fig 1) Cells allowed to stand in the saline medium for the same period of time without mechanical stimulation were considered as control preparations in resting conditions

When it was possible the control preparations and the stimulated preparations were dissected from the same animal and often from the same abdominal segment

The final preparation consisted of the standard specimen having the minimal dimensions still compatible with function (cell body of the neurone of the slowly adapting stretch receptor organ a segment of axon approximately 600 μ long and a 400 μ portion of the muscle bundle where the dendrites are imbedded) as described by Giacobini Handelman and Terzuolo (1963)

The holder containing the standard cell preparation was quickly immersed in a liquid [illegible]

inspection the samples appeared to be dried perfectly

[illegible]

wet weight)

Analytical methods

... into a 0.3 ml glass

Eastman and EDTA Merck

Results

Variations of glycolytic substrates and phosphate compounds

The results reported in Fig 2 are based on the analysis of 244 cells The glycogen level shows a linear decrease from the resting values of 5,040 ± 940 μmoles/kg w w to 2,740 ± 420 μmoles/kg w w after 22 × 10^4 impulses Conversely lactate showed an increase from 3,280 ± 600 μmoles/kg w w to 6,894 ± 545 μmoles/kg w w after 16 × 10^4 imp According to these results approximately 2 molecules of lactate were obtained for each one molecule of glycogen broken down These variations represent, in terms of resting values, a 67 per cent decrease for glycogen and 115 per cent increase for lactate (Fig 3)

The resting glucose level (1,931 ± 280 μmoles/kg w w) was not significantly altered after 16 × 10^4 imp (2,470 ± 278 μmoles/kg w w) As can be seen from the slope of the first part of the curve in Fig 2, there is a tendency to increase after further stimulation, that is after 16 × 10^4 imp It should however be pointed out that the level of glucose after total hydrolysis (N HCl 12 hrs at 120° C), not reported in Fig 2, shows a significant decrease from the resting values of 8,319 ± 430 μmoles/kg w w to 7,370 ± 560 μmoles/kg after 10 × 10^4 imp

As the lactate increases there is a similar increase in G6P from 79 ± 15 μmoles/kg w w at rest to 205 ± 29 μmoles/kg w w after 17 × 10^4 imp This variation of the G6P level is better seen when expressed in per cent values as in Fig 3

After stimulation ATP and ADP show variations of their respective levels in opposite directions ATP diminishes from 2,480 ± 565 μmoles/kg w w in resting conditions to 1,210 ± 134 μmoles/kg w w after 18 × 10^4 imp while ADP increases from 650 ± 240 μmoles kg w w at rest to 1,540 ± 354 μmoles/kg w w after 11 × 10^4 imp

After this, (Fig 2) ADP shows a tendency to decrease and when the cell has fired 16 × 10^4 imp it decreases to 1,144 ± 185 μmoles/kg w w In resting conditions the ratio ATP/ADP is 3 8, but after 11 × 10^4 imp this ratio approaches unity and the curves of phosphate compounds show a similar pattern

Inorganic phosphate increases from 8,410 ± 850 μmoles/kg w w at rest to 12,700 ± 650 μmoles/kg w w after 15 × 10^4 imp This represents a percentage increase of 51 per cent as shown in Fig 3

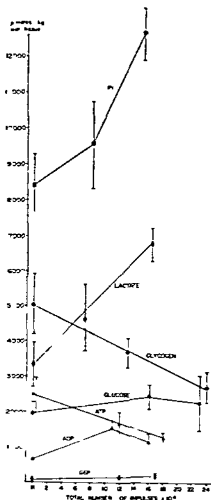


Fig. 2

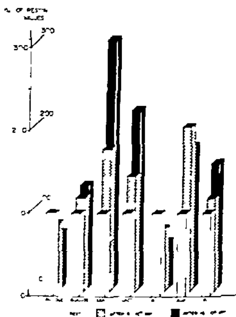


Fig. 3

Fig. 2. Variations of metabolites in the slowly adapting stretch receptor cell at rest and after prolonged physiological stimulation. Vertical bars represent ± S.E.

Fig. 3. Percentage changes in seven constituents of the slowly adapting stretch receptor cell after 8 × 10^4 and 16 × 10^4 impulses. The figure is based on averages values calculated from Fig. 2.

Variations of pyridine nucleotides

The oxidized and reduced forms of the pyridine nucleotides were studied in 104 cells. The mean values at rest and after 12 × 10^4 imp are reported in Table II. The table shows that after stimulation the ratio TPN/TPNH fell by more than 50 per cent. This was due to a decrease of TPN. Similarly the DPN/DPNH ratio fell by 50 per cent but in this case it was due to a decrease of DPN and a simultaneous increase of DPNH

TABLE II Variations of pyridine nucleotides in the slowly adapting stretch receptor cell at rest and after prolonged physiological stimulation

μmoles per kg wet weight	At rest	After 12×10^4 impulses
TPN	10 7±2 3	4 6±3 2
TPNH	15 4±1 4	14 6±0 6
TPN/TPNH	0 68	0 3
DPN	225±44	180±36
DPNH	58 1±6	90±14 8
DPN/DPNH	4	2

Discussion

The dynamic variations in the substrate levels after activity can best be seen in Fig 3 where the per cent variations from the resting state are reported It appears that the glycogen decreases consistently with the lactate increase The ratio between the glycogen consumed and the lactate formed is 0 5 after 12 × 10^4 imp These variations seem to suggest an increased glycolysis in the firing cell as compared with the control cell at rest Since the only experimental condition differing between the two groups of cells examined is the presence of impulse activity, it seems reasonable to assume that glycolysis is involved in some way with mechanism leading to impulse activity This assumption suffers from the obvious disadvantage that it attributes the variations of substrates levels exclusively to the mechanisms involved in the impulse activity within the neuronal components of the preparation Making the same assumption about the involvement of glycolysis the variation in the DPN/DPNH ratio during impulse activity and the significant increase in DPNH can be attributed to the higher activity of glycer aldehyde 3 phosphate dehydrogenase

In view of the role played by glucose in mammalian nervous system it is more difficult to explain the almost constant level after stimulation However if the crustacean nervous system follows the established pattern of glycolytic metabolism (Puyear Wang and Pritchard 1965) the almost steady level of glucose can be seen as a consequence of glycolytic control at the phosphorylase step This suggestion is supported by the significant increase of G6P in fact the highest *per cent change of the products* measured (Fig 3 An activated gluconeogenesis could also be a very important factor in such a mechanism

In view of these results the role played by pyruvate becomes of high interest both for the implications on the Krebs cycle and on the gluconeogenic processes (Whelan and Cameron 1964)

The continuous ATP decrease during prolonged stimulation justifies the previous assumption that this compound is a fundamental source of energy for transport systems involved in the impulse mechanism of this cell (Giacobini 1964)

While the ATP values are consistent with the variations of ADP the significance of the increase of Pi cannot be profitably discussed before the corresponding variations of AMP and arginine phosphate have been established This is now under investigation It should however be noted that the per cent variation of Pi is not particularly high in comparison with other metabolites (Fig 3), but on a molar basis our values appear to be relatively high

Summarizing our results it appears that they emphasize the importance of glycogen *as a source of energy* in the crustacean nerve cell which is in agreement with the earlier finding of Shanes and Brown (1942) in crab nerves The functional importance of glycogen for the impulse activity of the stretch receptor organ is also suggested by the fact that its concentration is higher in the fast adapting cell (8,439 $\pm$ 1,400 μmoles/kg w w) as compared to the slowly adapting cell (5,040 $\pm$ 940 μmoles/kg w w) Work is in progress to investigate the significance of glycogen in fast adapting activity

At the same time as the progress of our investigation optical studies of biochemical events in the electric organ of Electrophorus have been carried out by other investigators (Aubert Chance and Keynes 1964), and transients in glycolytic metabolism following electrical activity in the same organ have been studied by Maitra *et al* (1964)

The variations of glucose, G6P, lactate, ATP and ADP in the crustacean nerve cell after short lasting stimulation are similar to the pattern in the electric organ (Maitra *et al* 1964) Pi however first diminishes after 28 sec stimulation and increases following 4—5 sec recovery Large increases of fructose-1, 6-diphosphate, glyceraldehyde 3-phosphate 3 phosphoglyceric acid are found to occur at the time of the maximum fluorescence emission of the slices The fluorescence increase is identified with the DPN reduction at glyceraldehyde-3 phosphate dehydrogenase These authors conclude by suggesting that the activation of glycolysis may be due to a concerted effect of increased ADP and decreased ATP concentration upon phosphofructokinase, 1,3 diphosphoglycerate kinase and glyceraldehyde 3 phosphate dehydrogenase

Our results are in many aspects similar to the above and suggest that the impulse *activity in the crustacean nerve cell is linked to an energy requiring system* This energy is furnished by ATP splitting and glycolysis directly, or through the usual step of arginine phosphate supporting the ATP resynthesis The precise time relationship of these events can unfortunately not yet be elucidated by our experiments

[illegible] ional [illegible] d cal [illegible] ment [illegible] help [illegible] Dr J Passonneau and Dr L F Matschinsky (same department) was most useful for the analytical part of this work

References

AUBERT X B CHANCE and R D KEYNES Optical studies of biochemical events in the electric organ of electrophorus *Proc roy Soc* 1964 *B 160* 211—245

GERARD R W Nerve metabolism *Physiol Rev* 1932 *12* 469—592

GERARD R W and O MEYERHOF Untersuchungen uber den Stoffwechsel des Nerven *Biochem Z* 1927 *191* 125—145

GIACOBINI E, Neurophysiological and biochemical correlations in isolated nerve cell preparations at rest and during impulse activity *Abstr 2nd Meet Pharmacol (Prague)* Symposium on drugs and enzymes Edited by B B Brodie Part I Pergamon Press 1964 *38* 55—63

Giacobini E, E Handelman and C. Terzuolo Isolated neuron preparation for studies of metabolic events at rest and during impulse activity *Science* 1963 *140* 74—75
Grasso, A 1965 To be publ
Holmes E G Carbohydrates of crab nerve *Biochem J* 1929 *23* 1182—1186
Lorente de No R *A study of nerve physiology* Rockfeller Inst Med Res 1947 131 and 132
Lowry O H A quartz fiber balance *J biol Chem* 1941 *140* 183—186
Lowry, O H, The quantitative histochemistry of the brain *J Histochem Cytochem* 1953 *1* 420—427
Lowry, O H J V Passoneau D W Schulz and M K Rock The measurement of pyridine [illegible]

[illegible] 10—30
Maitra P K, A Ghosh B Schoener and B Chance, Transients in glycolytic metabolism following electrical activity in electrophorus *Biochem Bioph Acta* 1964 *88* 112—119
Puyear R L, Chick H Wang and A W Pritchard Catabolic pathways of carbohydrate [illegible] —153
[illegible] 3—4
[illegible] *Physiol*
[illegible]
Shanes A M and E. S Brown The effect of metabolic inhibitors on the resting potential of frog nerve *J cell comp Physiol* 1942 *19* 1—13
Strominger, J L. Enzymic synthesis of guanosine and cytidine triphosphates *Biochem Bioph Acta* 1955 *16* 616—618
Whelan W J and M P Cameron *Control of glycogen metabolism* Ciba Foundation Symposium Churchill Ltd 1964

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The Action of Tricarboxylic Acid Cycle Intermediates and Glutamate on the Impulse Activity and Respiration of the Crayfish Stretch Receptor Neurone

By

EZIO GIACOBINI and PIER CARLO MARCHISIO

Received 1 April 1965

Abstract

ketoglutarate (4 fold) succinate (2 8 fold) and malate (3 fold) Fumarate pyruvate and glutamate did not have any effect on the respiratory activity The only substrate showing an inhibitory effect on oxygen uptake was oxaloacetate Glucose tested for comparison also showed stimulatory effect (2 4 fold) on respiration The authors are inclined to believe that the effect on the impusle activity and respiration exerted by these compounds is confined to the nerve cell membrane The opposite modes of action of malate and oxaloacetate on respiration which did not affect impulse activity are however more difficult to explain in terms of a pure membrane mechanism

This paper is a report of part of a project in which we intend to investigate the physiological significance of the tricarboxylic acid cycle (TCA) in the crustacean stretch receptor neurone This cell can be readily isolated and maintained in apparently normal conditions for several hours Its electrical activity is easily recorded under direct inspection and its environment changed and controlled No other vertebrate or invertebrate nerve cell preparation is known which will satisfy these requirements It constitutes therefore a unique model for combined neuropharmacological and biochemical studies

In fact many substances have already been tested on this neurone, mainly in connection with the problem of GABA inhibition among these succinic and α ketoglutaric acids (Edwards and Kuffler 1959

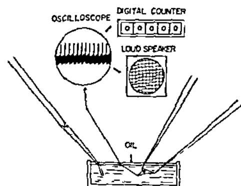


Fig 1 Stimulation technique, recording procedure and addition of known amounts of substrates to the isolated stretch receptor neurone

The tricarboxylic acid cycle is probably one of the major metabolic pathways in the crustacean nervous system, as it is in vertebrates (for ref see Wolvekamp and Waterman 1960, vol 1 and Puyear, Wang and Pritchard 1965). Carbohydrate metabolism seems to be a source of energy that is linked with the generation and propagation of nerve impulses in this neurone (Giacobini 1965, Grasso and Giacobini 1965)

It was for these reasons that we studied the effect of several intermediates of the TCA cycle on the impulse activity and respiration of the isolated crustacean neurone preparation Glutamate which is believed to interact with the same pathway (Haslam and Krebs 1963) was included in this study

Methods

Slowly adapting stretch receptor neurones from the first three abdominal segments of the [illegible] tic [illegible] of [illegible] va [illegible] 3331) (Fig 1) At the beginning of each experiment [illegible] to generat[illegible] minimal a[illegible] about 30 [illegible] each cell [illegible] to 50 μl depending on the effectiveness of the substrate.

In the respiration experiments the stretch receptor preparations were trimmed to the standard dimensions as previously reported by Giacobini *et al* (1963) and their functional activity tested The standard preparation was introduced into a cartesian diver and the oxygen uptake measured at rest and after addition of the substrate, according to the procedure described by Giacobini *et al* (1963) The concentrations of substrates for each group of experiments were chosen after taking into account results of the stimulation experiments

The substrate solutions were all prepared in van Harreveld from highly purified commercial sources and when necessary carefully adjusted to pH 7 All of the experiments were performed at 18—21° C The data here reported are obtained from experiments on more than 120 cells The substrates used with their source are listed below

Sodium citrate (British Drug Houses Ltd

Sodium isocitrate sodium α ketoglutarate sodium fumarate and sodium malate (Dr Theodor

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By

Ezio Giacobini and Pier Carlo Marchisio

Received 1 April 1965

Abstract

Giacobini, E. and P. C. Marchisio. *The action of tricarboxylic acid cycle intermediates and glutamate on the impulse activity and respiration of the crayfish stretch receptor neurone.* Acta physiol. scand. 1966. *66.* 58—66. — The effect of seven intermediates of the TCA cycle, of pyruvate and glutamate was studied on the impulse activity and oxygen uptake of the slowly adapting cell of the stretch receptor organ of crayfish. Citrate, isocitrate, α-ketoglutarate and succinate showed a strong excitatory action, fumarate, malate, oxaloacetate and pyruvate had no action while glutamate had an inhibitory action on the impulse activity. The most effective substrate tested was citrate which also increased respiration sevenfold. Isocitrate showed a lower effect (2.2 fold) as did α-ketoglutarate (4 fold), succinate (2.8 fold) and malate (3 fold). Fumarate, pyruvate and glutamate did not have any effect on the respiratory activity. The only substrate showing an inhibitory effect on oxygen uptake was oxaloacetate. Glucose, tested for comparison, also showed stimulatory effect (2.4 fold) on respiration. The authors are inclined to believe that the effect on the impulse activity and respiration exerted by these compounds is confined to the nerve cell membrane. The opposite modes of action of malate and oxaloacetate on respiration, which did not affect impulse activity, are however more difficult to explain in terms of a pure membrane mechanism.

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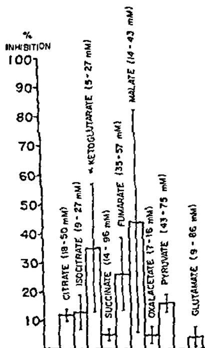


Fig 4

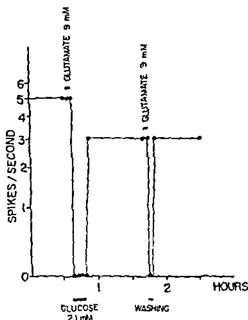


Fig 5

Fig 4 The effect of tricarboxylic acid cycle intermediates and glutamate on the spike height of the stretch receptor neurone Each bar represents the mean of at least 10 experiments (± C L 95 %)

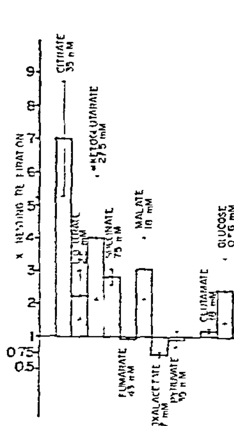
Fig 5 A typical experiment showing the effect of glutamate on the impulse frequency of the stretch receptor neurone The glutamate block is reversed by glucose and by washing

to the initial value (Fig 3) The effective concentrations of citrate ranged from 18 to 50 mM EDTA (18 mM) mimicked, almost perfectly, the effect of citrate The height of the spike was not changed significantly by citrate (Fig 4)

Isocitrate this substrate increased impulse activity by a factor of 12 7 (Fig 2), the maximum frequency recorded being about 100 imp sec Isocitrate stimulation showed features similar to the citrate effect the impulse frequency decreasing very quickly after having reached a maximum and finally becomes a block There then followed a period of alternating recovery and block, the interval between the bursts becoming progressively longer and longer and the duration of the burst shorter until a permanent block of activity was produced This period lasted from a few minutes up to 3 hrs and was generally reversible by washing with fresh physiological solution

There was no noticeable effect on the height of the spike after application of isocitrate (Fig 4)

α-ketoglutarate this substrate showed the least effect among the excitatory substrates (Fig 2) increasing the impulse activity to about twice the initial rate This high frequency level was maintained for several hours The effect reported was observed with concentra-



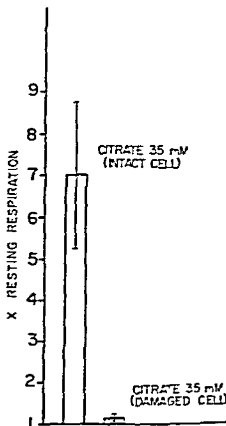


Fig 6 Fig 7

Fig 6. The effect of tricarboxylic acid cycle intermediates, glutamate and glucose on the respiration of the stretch receptor neurone. Each bar represents the mean of the ratios (± C.L. 95 %) between the oxygen uptake in the presence of the substrate and the control resting respiration.

Fig 7. The action of 35 mM citrate on intact cells and on mechanically damaged cells. Each bar represents the mean of the ratios (± C.L. 95 %) between the oxygen uptake in the presence of the substrate and the control resting respiration.

tions ranging between 5 and 27 mM. The spike height was significantly decreased by 35 per cent (Fig. 4).

Succinate increased the firing frequency by more than 15 times the initial as reported in Fig. 2. The maximal frequency reached was about 100 impulses per second. The same blocking effect, already described for citrate and isocitrate, was observed. Washing restored the initial activity. In these experiments the spike height was virtually unchanged for several hours (Fig. 4).

Fumarate, malate, oxaloacetate and pyruvate did not show any effect on the impulse frequency in spite of the fact that some of the cells were exposed to increasing concentrations for periods up to 8 hrs (Fig. 2). Fumarate and malate, in most experiments, decreased the height of the spike significantly (Fig. 4).

Glutamate L-glutamate was the only substrate showing a constant and powerful inhibitory effect on the impulse activity, block occurring immediately after the addition of this substrate (Fig 5) Our recording technique was unable to detect an excitatory phase preceding this inhibitory effect After L-glutamate inhibition there was no spontaneous recovery of impulse activity, but the application of glucose (1 mM) or washing instantaneously restored the initial activity There was a great degree of individual variation among these cells, the inhibiting concentration varying as much as tenfold between different cells No marked effect was noticed on spike height (Fig 4) before the blocking concentration was reached

b) *Experiments on the respiratory activity*

The experiments in which the oxygen uptake of the crustacean neurone was measured before and after adding the substrate are summarized in Fig 6 This figure shows the ratios of oxygen uptake in the presence of each substrate compared to control resting respiration Each bar represents the mean of several experiments The most effective substrate tested was citrate which increased respiration 7-fold Isocitrate showed a lower effect (2 2 fold) as did α ketoglutarate (4 fold), succinate (2 8 fold) and malate (3 fold) Fumarate, pyruvate and glutamate did not have any effect on the respiratory activity The only substrate showing an inhibitory effect was oxaloacetate which decreased the oxygen uptake to 73 % of the control

Glucose, tested for comparison, also showed a stimulatory effect (2 4 fold) at a concentration of 0 56 mM which has been shown to restore impulse activity after metabolic inhibition (Giacobini 1965)

A final series of experiments was carried out with normal cells and with cells mechanically injured by gently crushing their axon hillocks with fine forceps The latter cells, because of the localized membrane damage, were no longer capable of firing impulses As shown in Fig 7 citrate (35 mM) stimulated the respiration of intact cells but had no effect on the injured cells

Discussion

The effect of naturally occurring substrates added to the environment of nerve cells can be interpreted as a 'pharmacological' effect at the level of the nerve cell membrane, or as a 'metabolic' effect due to a direct interaction of these substances with the cell metabolism generally or specifically with energy processes linked to impulse activity The 'pharmacological' interpretation is supported by the works of those authors who investigated the effects of iontophoretically applied substrates on single CNS [illegible]

inhibitory transmitter

L-glutamic acid has been found to be strongly excitatory for spinal neurone activity (Curtis Phillis and Watkins 1959 1960, Curtis and Watkins 1960, 1963) A similar effect had been earlier reported by Hayashi (1954) and by Purpura *et al* (1959) Krnjevic and Phillis (1961 1963) found that L-glutamic acid first excites cortical neurones but after prolonged administration produced an overdepolarization with a subsequent cathodal block

In crustacea Elliot and Florey (1956) were unable to detect any effect of L-glutamate on the stretch receptor neurone It is however well established that glutamate excites

crustacean muscles (Robbins 1959, van Harreveld and Mendelson 1959) More recently Takeuchi and Takeuchi (1964) found that iontophoretically applied glutamate acts on muscle receptor sites which can be identified as neuroreceptors Our results do not suggest an excitatory action for glutamate since we observed an immediate and complete block of the impulse activity, but this effect could be due to a mechanism of cathodal block similar to that observed by Krnjevic and Phillis (1963) Another explanation could be a direct inhibition similar to that reported by Gerschenfeld and Lasansky (1964) on snail central neurones Unfortunately as our recording technique is rather simple, it does not allow us to analyse in greater detail the mechanism of action of this substance

Whatever the explanation may be, the action of glutamate seems to be confined to the cell membrane Glutamate *in fact is not likely to modify the impulse activity* through the cell metabolism, as intracellularly injected glutamate is without effect (Coombs, Eccles and Fatt 1955, Araki *et al* 1961, Takeuchi and Takeuchi 1964) However, it should be noted that glutamate increases the intracellular K^+ (Hillman and McIlwain 1961) although this fact is not easily correlated with our findings so far

Citrate, isocitrate, α ketoglutarate and succinate, the 4 TCA intermediates demonstrated to affect impulse activity could act by a membranal mechanism α ketoglutarate and succinate had previously been shown to increase impulse activity in the crayfish stretch receptor (Edwards and Kuffler 1959) It is noteworthy that these substrates have the capacity of forming weakly dissociated calcium salts and that diminution of the extracellular Ca concentration will elicit spontaneous discharge (Brink 1954) The hypothesis that a diminution of calcium ions is involved, is supported by the observation that EDTA mimics citrate action and that citrate block is suppressed by adding calcium ions or by replacing the bathing medium with fresh van Harreveld solution The same results are observed with the other three intermediates Corriol Joanny and Millet 1964 describe a similar action of Ca free solutions or of added EDTA in cerebral cortex slices

The metabolic interpretation suffers from the uncertainty that it is not known whether the substrates employed permeate the cell membrane In fact we have no evidence that they are freely permeable and can reach the metabolic loci inside the cell If the changes have a metabolic origin it would perhaps be less likely to exhibit an immediate recovery after washing We are therefore inclined to believe that the effect on the impulse activity exerted by these compounds is confined to the membrane

The same considerations apply to the respiration experiments but in this case there is strong evidence for a metabolic action of several intermediates Different nervous tissue preparations have been used varying from brain mitochondrial fractions of Brody and Bain 1952 to the cortex slices of Livingstone *et al* (1962) and Haslam and Krebs (1963 The action of some TCA cycle intermediates on the respiration of single Deiters cells has been investigated by Hamberger (1961) but no work has previously been carried out with nerve cells which are isolated and physiologically capable of responding to stimulation There is therefore the difficulty of correlating results from experiments on these vastly different preparations It is possible that the increase in oxygen consumption supported by the addition of citrate isocitrate α ketoglutarate and succinate is due to their effect on the impulse activity If this is the case it confirms the previous data indicating a relationship between oxygen consumption and impulse activity (Giacobini *et al* 1963 1965 Giacobini 1964

Taking into account the Ca binding effect of some of the substrates, the fact that

nerve tissue respiration may be stimulated by the reduction of extracellular calcium without the presence of spontaneously initiated impulse activity (Brink *et al* 1946, Brink 1954) is not supported by our results In fact the oxygen uptake of the crustacean nerve cell in Ca + free solutions has been found to be markedly diminished (Giacobini 1964 Giacobini *et al* 1965) All of the TCA cycle substrates have been found to support the respiration of brain mitochondria (Brody and Bain 1952) especially pyruvate and malate Livingstone *et al* (1962) claim that pyruvate oxaloacetate and α ketoglutarate support the respiration of rat brain slices at the same rate as glucose, while malate fumarate and citrate are less effective than glucose In our experiments only malate stimulated the respiration more than glucose, whereas fumarate, pyruvate and glutamate did not increase the resting respiration significantly The fact that glutamate does not increase the oxygen uptake of the crustacean neurone contrasts with the results on isolated vertebrate nerve cells (Hamberger 1961) In our study oxaloacetate was the only substrate shown to cause a significant decrease in resting respiration

The respiratory stimulation by substrates activating impulse activity can be explained in terms of an augmented need for energy supplied through oxidative processes The modes of action of malate and oxaloacetate which do not affect impulse activity are more difficult to explain in terms of a pure membrane mechanism If malate and oxaloacetate are to be supposed to act through a metabolic' mechanism involving the TCA cycle of the crustacean neurone one needs to postulate opposing effects for these substrates Brody and Bain (1952) found that malate associated to pyruvate supports the highest oxygen uptake in brain mitochondria The importance of the different steps of the TCA cycle in connection with the generation and propagation of the impulse can be better analysed by determining the subsequent variations of the substrate levels after physiological stimulation For this reason we are now determining the levels of the substrates which have been subject of this study using the microchemical methods described in Lowry's laboratory (1964)

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References

ARAKI T M ITO and O OSCARSSON Anion permeability of the synaptic and nonsynaptic motoneurone membrane *J Physiol* (Lond) 1961 (Lond.) *159* 410—435

BERL S and H WAELSCH Determination of glutamic acid, glutamine glutathione and γ aminobutyric acid and their distribution in brain tissue *J Neurochem* 1958 *3* 161—169

BRINK F The role of calcium ions in neural processes *Pharmacol Rev* 1954 *6* 243—298

BRINK F D W BRONK and M G LARRABEE Chemical excitation of nerve *Ann New York Acad Sci* 1946 *47* 457—485

BRODY T M and J A BAIN Mitochondrial preparation from mammalian brain *J biol Chem* 1952 *195* 685—696

COOMBS J S J C ECCLES and P FATT The specific ionic conductances and the ionic movement across the motoneuronal membrane that produce the inhibitory postsynaptic potential *J Physiol* (Lond) 1955 *130* 326—373

CORRIOL J P JOANNY and A MILLET Gradients ioniques alcalins et respiration du cortex cérébral isolé de mammifère Role du glucose effect de la stimulation electrique et de divers agents de stimulation métabolique *Arch int Physiol* 1964 *72* 615—631

CURTIS D R J W PHILLIS and J C WATKINS The depression of spinal neurones by γ-amino-n butyric acid and α alanine *J Physiol* (Lond.) 1959 *146* 185—203

CURTIS, D, J W PHILLIS and J C WATKINS The chemical excitation of spinal neurones by certain acidic amino acids *J Physiol* (Lond) 1960 *150* 656—682

CURTIS, D and J C WATKINS The excitation and depression of spinal neurones by structurally related amino acids *J Neurochem* 1960 *6* 117—141

CURTIS, D and J C WATKINS Acidic amino-acids with strong excitatory actions on mammalian neurones *J Physiol* (Lond) 1963 *166* 1—14

GERSCHENFELD, H M and A LASANSKY, Action of glutamic acid and other naturally occurring amino acids on snail central neurons *Int J Neuropharmacol* 1964 *3* 301—314

GIACOBINI E, The effect of metabolic and ion transport inhibitors on the impulse activity and oxygen uptake of an isolated crustacean neurone *Acta physiol scand* 1966 *66* 34—48

HAMBERGER A, Oxidation of tricarboxylic acid cycle intermediates by nerve cell bodies and glial cells *J Neurochem* 1961 *8* 31—35

VAN HARREVELD A Compounds in brain extracts causing spreading depression of cerebral cortical activity and contraction of crustacean muscle *J Neurochem* 1959 *3* 300—315

VAN HARREVELD A A physiological solution for freshwater crustaceans *Proc Soc exp Biol* (N Y) 1936 *34* 428—432

VAN HARREVELD A and M MENDELSON Glutamate induced contractions in crustacean muscle *J cell comp Physiol* 1959 *54* 85—94

HASLAM R J and KREBS H A The metabolism of glutamate in homogenates and slices of brain cortex *Biochem J* 1963 *88* 566—578

HAYASHI T Effects of sodium glutamate on the nervous system *Keio J Med* 1954 *3* 183—192

HILLMAN H H and H McILWAIN Membrane potentials in mammalian cerebral tissues in vitro dependence on ionic environment *J Physiol* (Lond) 1961 *157* 263—278

KRNJEVIC K and J W PHILLIS The action of certain amino acids on cortical neurones *J Physiol* (Lond 1961 *159* 62—63

KRNJEVIC K and J W PHILLIS Iontophoretic studies of neurones in the mammalian cerebral cortex *J Physiol* (Lond) 1963 *165* 274—304

LIVINGSTON A C H WILLIAMS and F W BARNES JR Augmentation of oxygen consumption of rat brain in vitro by various carbohydrate intermediates *Proc Soc exp Biol* (N Y) 1952 *111* 75 78

LOWRY O H *Microanalysis for histochemical purposes* Second int Congr Histo- and Cytochem (Francfurt Main) Springer Verlag Berlin 1964 62—69

PURPURA D P M GIRADO T G SMITH D A CALLAN and H GRUNDFEST Structure activity determinants of pharmacological effects of amino acids and related compounds on central synapses *J Neurochem* 1958 *3* 238—268

PUYEAR R L C H WANG and A W PRITCHARD Catabolic pathways of carbohydrate in the intermolt crayfish Pacifastacus Leniusculus *Comp Biochem Physiol* 1965 *14* 145—153

ROBBINS J The excitation and inhibition of crustacean muscle by aminoacids *J Physiol* (Lond) 1959 *148* 39 50

SINGH S I and G I MALHOTRA Amino acid content of monkey brain I) General pattern and quantitative value of glutamic acid/glutamine aminobutyric acid and aspartic acid *J Neurochem* 1962 *9* 37—42

TAKEUCHI, A and N TAKEUCHI The effect on crayfish muscle of iontophoretically applied glutamate *J Physiol* (Lond 1964 *170* 296—317

TOWER D B *Neurochemistry of epilepsy* C C Thomas Springfield III 1960 p 61

WOLVEKAMP, H P and T H WATERMAN in Waterman T H *The physiology of crustacea* Vol I Academic Press (New York and Lond 1960

Acta physiol. scand. 1966. 66. 67—71

From the Department of Pharmacology, University of Gothenburg Sweden

Lactate Elimination and Oxidation in Thyroidectomized, Untreated and Thyroxine-treated Rabbits

By

NILS SVEDMYR

Received 27 April 1965

Abstract

SVEDMYR, N. *Lactate elimination and oxidation in thyroidectomized, untreated and thyroxine-treated rabbits.* *Acta physiol scand* 1966 *66* 67—71 — On the infusion of 11.5 mg L(+)lactate per kg per min for 60 min in the rabbit the increase in the lactate concentration in the blood was smaller in thyroxine-treated than in untreated and thyroidectomized animals This was considered to be due to an increase in the metabolic rate of lactate after thyroxine The regression coefficient for the correlation between the stimulation of oxygen consumption and the increase in the lactic acid content of the blood was higher in thyroxine-treated than in untreated and thyroidectomized animals

The increase of the lactic acid content of the blood during infusion of adrenaline was decreased in the dog (Brewster *et al* 1956), and in the rabbit (Svedmyr 1965) after thyroidectomy and increased after thyroxine treatment The reason for the increase in the adrenaline effect after thyroxine may have been that the rate of lactic acid production in the tissues was increased and/or that the elimination and oxidation of lactic acid were decreased In an attempt to differentiate between these alternatives experiments were performed in which L(+)lactate was infused in a constant dose into thyroidectomized, untreated and thyroxine-treated rabbits, and it was studied how this infusion affected the lactic acid content of the blood and the oxygen consumption

The naturally occurring lactate isomer was used since L(+)lactate is metabolised approximately 4 times more rapid than D(—)lactate (Lundholm 1958)

Method

In principle the experiments were performed as described previously (Svedmyr 1965) The animals had undergone thyroidectomy at least 6 weeks before the experiment The animals in the thyroxine-treated group were given 1 mg L-thyroxine (Levaxin®, Nygaard) per day subcutaneously for 7 days prior to the experiment The "untreated" animals were injected with the solution medium alone Before the experiment the animal were starved for 18 hrs. A

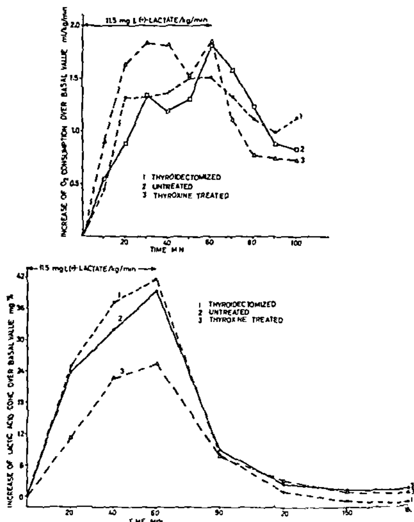


Fig 1 The effect of infusion of 11 5 mg L(+)lactate as sodiumsalt, on the oxygen consumptio and on the lactic acid content of the blood in untreated thyroidectomized and thyroxin treated rabbits Mean increase over basal value of 5 experiments

cannula was introduced into the central artery of one ear, under local anesthesia for the with

TABLE I The effect of intravenous infusion of 11.5 mg L (+) lactate per kg per min for 60 min on the lactic acid content of the blood. Test on untreated, thyroidectomized and thyroxinetreated rabbits

P = probability that the effect was due to chance. n = number of tests

	Mean weight kg	Basal values mg/100 ml	Increase after adrenaline mg per cent 20	40	60	90	120
Untreated n = 5	2.83	4.8 ± 0.9	23.9 ± 3.4	32.0 ± 3.9	39.4 ± 3.5	8.9 ± 1.6	2.5 ± 1.0
Thyreoidectomized n = 4	3.31	5.9 ± 1.1	25.1 ± 4.7	37.2 ± 4.2	41.6 ± 6.9	8.1 ± 2.5	1.0 ± 1.7
Thyroxinetreated n = 5	2.75	4.8 ± 0.5	11.4 ± 3.3	22.7 ± 3.6	25.4 ± 2.4	7.8 ± 3.6	3.0 ± 0.8
Difference between							
Thyreodectomized — untreated			1.5 ± 5.9	5.2 ± 5.7	2.2 ± 7.6	−0.8 ± 3.0	−1.5 ± 1.6
Untreated — thyroxinetreated			12.2 ± 4.9 P < 0.05	9.3 ± 4.3 P < 0.01	14.0 ± 4.2 P < 0.02	1.0 ± 2.5	−0.5 ± 1.2

of the infusion, and the oxygen consumption was also recorded at the same time. The lactic acid content was determined on 1.5 ml blood as according to Friedemann and Graeser (1933). The lactic acid excretion in the urine was insignificant which agreed with the results of Dietrich and Zeyen (1932).

Results

The effects of lactate infusion on the content of lactic acid in the blood in untreated, thyroidectomized and thyroxine treated animals are shown in Fig. 1 and Table I. The lactic acid content of the blood reached a maximum after 60 min infusion and after 120 min had reached the original value. The increase in the lactic acid content during the infusion period was somewhat higher in the thyroidectomized than in the untreated animals but the difference was not statistically significant (Table I). In the thyroxine treated animals the increase in the lactic acid content during the infusion was considerably smaller than in the untreated animals and these differences were statistically significant. The summed mean difference after 20, 40 and 60 min was 11.8 ± 2.60 ($P < 0.001$).

These results indicated that the animals treated with thyroxine were able to metabolise the infused lactate more rapidly than the untreated animals. Part of the lactate administered (about one sixth) probably became oxidized while the rest was metabolized in other ways (Lundholm 1949). It was therefore of interest to determine

TABLE II The influence of L (+) lactate infusion on the oxygen consumption of untreated thyroidectomized and thyroxinetreated rabbits Increase in oxygen consumption was measured 0—60 min after the start of the infusion n=number of tests

		Basal oxygen consumption ml/kg/min	Increase in O_2 consumption in ml/kg/min over basal values during infusion of L (+) lactate
Untreated	n=5	8 56±0 24	1 34±0 17
Thyreoidectomized	n=4	7 20±0 11	1 24±0 15
Thyroxinetreated	n=5	11 00±0 27	1 59±0 41
Difference between			
Untreated — thyroidectomized		1 34±0 26 P< 0 005	0 10±0 22
Thyroxinetreated — untreated		2 44±0 36 P< 0 001	0 25±0 45

TABLE III Correlation between increase in oxygen consumption in ml/kg/min (Y) over the basal value and increase in lactic acid content (X) of the blood in mg per cent P=probability that the effect was due to chance n=number of tests

	Regression equation	Coefficient of correlation
Untreated n 36	Y 0 029 X + 0 62	0 756 P< 0 001
Thyroidectomized n 30	Y = 0 026 X + 0 44	0 709 P< 0 001
Thyroxine treated n 36	Y = 0 045 X + 0 43	0 510 P< 0 005

whether the increase in O_2 consumption during the lactate infusion varied in the different experimental groups The effects on the O_2 consumption are shown in Fig 1 and Table II The increase in the O_2 consumption was about 20 % larger during the infusion period in the thyroxine treated than in the untreated animals but the difference was not statistically significant

A more quantitative comparison of the increase in the lactate content and the increase in O_2 consumption was made however in the different groups The increase of the O_2 consumption above the basal value in ml/kg/min during the 10 min period in which the blood sample was taken has been correlated in Table III with the increase in the lactic acid content of the blood above the basal value As may be seen this correlation was statistically significant in all of the experimental groups but the value of the regres-

sion coefficient in the group of thyroxine-treated animals was considerably higher than in the untreated and thyroidectomized animals This may indicate that with an equal increase in the lactic acid content in the blood, the O_2 consumption had increased more in the thyroxine treated animals than in the other groups

Discussion

The experiments described above showed that the elimination of infused lactate was more rapid in thyroxine treated than in untreated and thyroidectomized animals In the cases in which the lactic acid content after adrenaline infusion was increased after thyroxine treatment (Svedmyr 1965), this was probably due to an increased production and not a decreased elimination of lactate This applies at least to more moderate degrees of thyroxine treatment The increased elimination of lactate may be due at least in part to more rapid lactic acid oxidation, since the regression coefficient between the increase in O_2 consumption and the increase in the lactate content of the blood was larger after thyroxine treatment

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References

BREWSTER W R Jr J P ISAACS P F OSGOOD and T L KING The hemodynamic and metabolic interrelationship in the activity of epinephrine norepinephrine and the thyroid hormones *Circulation* 1956 *13* 1—20

DIETRICH S and M ZEYEN Quantitative Untersuchungen uber die Resynthese der Milchsaure beim Menschen *Kl n Med* 1932 *120* 517—525

FRIEDEMANN T E and J B GRAESER The determination of lactic acid *J biol Chem* 1933 *100* 291—308

[illegible]

LUNDHOLM L E MOHME LUNDHOLM and N SVEDMYR Comparative investigation of methods for determination of lactic acid in blood and tissue extracts. *Scand J clin Lab Invest* 1963 *15* 311—316

SVEDMYR N The influence of thyroxine treatment and thyroidectomy on the calorigenic and some other metabolic effects of adrenaline and noradrenaline in experiments on fasted rabbits *Acta physiol scand* 1966 *66* In press

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From the Neurophysiological Laboratory at the Anatomical Institute, University of Oslo, Norway

Peripheral Autonomic Influence on the Motility of the Urinary Bladder in the Cat

II. 'Tone'

By

RAGNAR GJONE

Received 28 April 1965

Abstract

GJONE, R. *Peripheral autonomic influence on the motility of the urinary bladder in the cat. II 'Tone'.* Acta physiol. scand. 1966. *66.* 72—80. — Parasympathetic and sympathetic influence on the 'tone' of the bladder was revealed through alterations in the slope of the pressure-volume records (cystometrograms) following partial and complete section of the autonomic nerve supplies to the vesical wall. When all bladder nerves but the efferent parasympathetic fibres were cut bilaterally, a marked elevation of vesical tone was noted. By additional section of the efferent parasympathetic supply (total extrinsic denervation) this increase in the tone of the bladder was completely abolished. A moderate decrease in vesical tone primarily ensued by section of the parasympathetic supply alone, leaving the sympathetic innervation intact. After additional sympathetic denervation, thereby depriving the bladder of all extrinsic nerves, vesical tone again increased close up to normal levels. Thus, the tone of the urinary bladder was shown to be under influence of excitatory parasympathetic and inhibitory sympathetic effects.

The term 'tone' of the urinary bladder has been widely used in the past to characterize the slope of the intravesical pressure-volume curve during bladder distension. Already Mosso and Pellacani (1882), who introduced systematic pressure-volume determinations (cystometry) in bladder studies, analysed the curves obtained in terms of vesical tone. In order to maintain renal excretion the intravesical pressure remains low during urine accumulation up to the micturition threshold. This property of the normal bladder also appears from the cystometrograms which are traditionally produced by filling the bladder step by step. After each volume increment an initial sharp elevation of the intravesical pressure is noted, followed by a gradual decline towards the pre-filling pressure level. The latter phase of the pressure response has been referred to as "adaptation", whereby a nervous regulation of vesical tone is suggested. Thus, Sherrington

(1915) regarded the tone of the bladder as a reflex phenomenon comparable to the myotatic reflexes of skeletal muscles According to the view most widely accepted in the past, bladder tone is subordinated nervous influence from the supraspinal level Inhibitory effects from the cerebral cortex and the diencephalon are assumed to counteract excitatory centers" within the brain stem and the spinal cord (Stewart 1900, Elliott 1907, Holmes 1933, Munro 1935, Evans 1936, Langworthy, Kolb and Lewis 1940, Prather 1953, Kollberg and Petersén 1963) Following transection of the spinal cord these investigators recorded bladder atonia", which during the next weeks or months was replaced by a hypertonic state The temporary loss of bladder tone was considered due to 'spinal shock", whereas the increased tonus observed later was explained as a release phenomenon characterized by liberation of excitatory effects mediated from a sacral autonomic 'center" Denny-Brown and Robertson (1933) found that intravesical pressure adaptation was present also in patients with lesions of the cauda equina Based on systematic studies of the late stages of such cases they concluded that the tone of the bladder is regulated by excitatory and inhibitory nervous effects, exerted from autonomic ganglia lying in the vesical wall

None of these investigators have obtained any evidence which differentiates parasympathetic effects from those exerted by the sympathetic supply In general, the sympathetic nerves were assumed to play at most a subordinate role in the regulation of bladder tone

In contrast to the traditional concepts cited above, more recent workers (Nesbit *et al* 1947, Nesbit and Lapides 1948, Carpenter and Root 1951, Langley and Whiteside 1951, Tang and Ruch 1955 and Ruch 1960) have advanced the opinion that the tone of the bladder is non-neurogenic in nature, reflecting the physical state of the vesical wall only This conclusion was based mainly upon the results of cystometric examinations in the spinal or decerebrate state as compared with those obtained in the intact animal

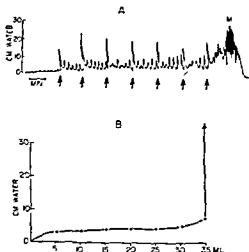
In a preceding study (Gjone 1965) the rhythmic bladder activity was shown to be modulated by an excitatory parasympathetic and an inhibitory sympathetic influence The present investigation was performed in order to clarify whether a similar dual nervous mechanism is regulating the tone of the bladder as well The influence exerted through the parasympathetic and sympathetic pathways was analysed separately by cystometric examinations under step-wise interruption of these autonomic nerves In particular, the demonstration of inhibitory sympathetic effects on bladder tone was aimed at

Material and methods

S[illegible] cystometrograms were obtained in 26 adult cats males and females (weight 1 9—4 3 [illegible]

level of anesthesia [illegible]

the pressure transducer was ordinarly shut during injection of fluid then immediately opened for recording the pressure responses to each additional volume increment The fluid



the three-way valve connecting the bladder cavity to the pressure transducer Tube connection to the transducer shut during injection, then opened for intravesical recording M, micturition *B* Graphic reproduction of the cystometrogram in A by plotting prefilling pressure levels (in cm water) against volume (in ml). (Cat 4)

introduced was warmed so as to accord with the animal's body temperature Slight changes (± 2° C) in the temperature of the saline solution did not interfere with the results obtained inasmuch as even temperature variations from 10 to 45° C did not (according to a single set of observations in a control experiment) induce any essential effects on the pressure responses to gradual bladder distension

Progressive interruption of the autonomic nerve supply to the bladder, was achieved by the operative procedures described in a preceding communication (Gjone 1965)

Four animals were kept alive for a period of 10—14 days after inserting the catheter in the vertex region At intervals of 3—4 days cystometric examinations were carried out, whereby alterations in tone during the development of a cystitis could be observed

Results

Having established a continuous intravesical pressure recording, cystometrograms were obtained in all animals prior to any interference with the vesical nerve supply The initial cystometrograms in the 26 cats studied revealed variations in the micturition threshold *ranging from 10 to 70 ml With respect to the intravesical pressure level* recorded at equal volumes differences occurred, but to a minor extent, and a striking conformity was found to characterize the slope of the pressure-volume diagram ("the tonus curve" provided the experimental conditions were essentially the same from cat to cat A representative pressure curve is shown in Fig 1, from which the following typical features appear (i) An initial pressure elevation caused by each volume increment, succeeded by (ii) a gradual decline toward a resting pressure level (iii) By *plotting the pre-filling pressure levels (in cm water) against bladder volumes (in ml)* a graphic representation of the pressure-volume curve is obtained (Fig 1 B), showing that the resting pressure remains relatively constant up to a critical degree of bladder filling ("the micturition threshold Then a steep rise of the intravesical pressure indicates the initiation of micturition (iv) Superimposed on the slope of the pressure-volume record (cystometrogram rhythmic pressure fluctuations appear, which also remain fairly constant in amplitude 5—10 cm water) as well as in frequency (3—4 per min) up to the point of micturition This is preceded by a rapid increase of the spontaneous bladder contractions particularly with respect to their frequency

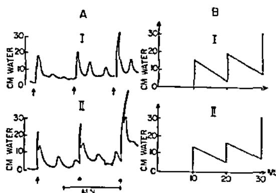


Fig 2 *A* Cystometrograms obtained from cat with intact bladder innervation I Tube connecting the vesical cavity with transducer shut during injection (10 ml at arrows) then opened for pressure recording II Tube to the transducer open also during injection (10 ml at arrows) *B* Graphic reproduction of the two cystometrograms I Resting pressure levels and post filling pressure peaks plotted against volume II Resting pressure levels and the *second* pressure peak following each injection plotted against volume The first peak is caused by the injection pressure (Cat 91)

In Fig 2 two cystometrograms are shown, which enable a more detailed analysis of the initial pressure peak occurring subsequent to each volume increment Firstly, stepwise bladder filling was performed in the ordinary way (I) *i e*, the tube connecting the bladder to the transducer was closed during the injection then immediately opened for intravesical pressure recording Then another cystometrogram was obtained in the same animal with the connecting tube open during injection (II) Here two pressure peaks are seen to follow each volume increment, the first of which is merely reflecting the injection pressure The second rise in the intravesical pressure level appearing after a latency of 2—3 sec represents the *dynamic* or early response to stretch as seen from the graphic reproduction of these cystometrograms (Fig 2 B)

minations were performed A higher bladder tone associated with a lower micturition threshold was found in all experiments under chloralose-urethane than after the additional injections of nembutal (Fig 3 A and B)

Effects of inflammatory changes in the vesical wall Changes in vesical tone were demonstrated by the cystometrograms obtained at different stages of a developing cystitis in 4 cats The animals were given the same type of anesthesia and in equal doses before each cystometric examination which was performed at intervals of several days The records resulting from the first pressure volume determination (2 hrs after initiating anesthesia) were compared A gradual rise in bladder tone associated with decrease of micturition threshold thereby ensued as illustrated in Fig 4 A and B

The presence of a cystitis was verified at autopsy whereby marked inflammatory changes associated with a thickening of the vesical wall were found

Effects of nerve sectioning

In 18 animals bilateral section of the autonomic bladder nerves was carried out step by step

1 *Initial section of the parasympathetic nerves* was performed in 6 cats Apart from a marked reduction of the rhythmic bladder contractions (Gjone 1965 this procedure

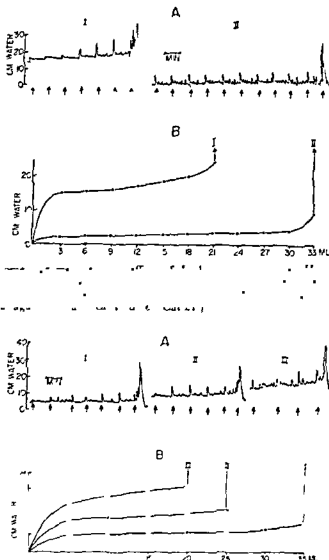
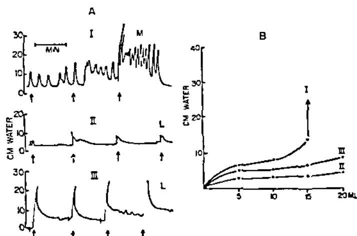


Fig 4 Alteration in the slope of the pressure volume record during a developing cystitis caused by an indwelling catheter see text *A* Cystometrograms obtained on the day of operation (I) five (II) and ten days later (III) respectively Volume increments of 5 ml indicated by arrows *B* Graphic representation of the three cystometrograms by plotting the pre filling pressure level (in cm of water) against the corresponding intravesical volume (in ml) (Cat 22)

was followed by the abolition of the micturition reflex and by a slight lowering of the tonus curve After additional section of the sympathetic vesical supply the slope of the cystometrogram was again changed so as to reflect nearly normal bladder tone (Fig 5

2 *Interruption of the sympathetic nerves* 8 animals) maintaining the parasympathetic innervation intact, caused a prompt and profound reduction in the micturition threshold



bladder again increased although complete normalization is not seen The micturition reflex still absent. *B* Graphic reproduction of the three cystometrograms (Cat 77)

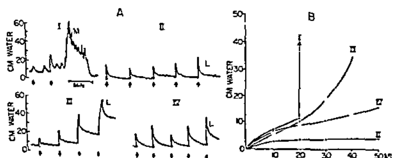


Fig 6 A Effects of progressive bladder denervation on the cystometrogram M, regular

As a rule micturition was induced already by the first 2—4 ml injected into the bladder Hence, with the direct approach to the vesical cavity, leaving the emptying function completely intact cystometrograms, suitable for comparing tonus changes could not be obtained In order to meet this requirement, the denervation procedure was initiated in 4 cats by cutting the afferent sacral nerves thereby abolishing the micturition reflex

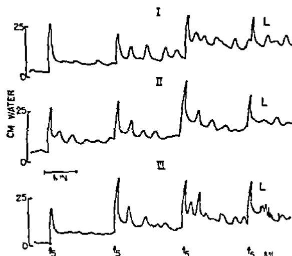


Fig 7 Three successive cystometrograms after section of all extrinsic bladder nerves Volume increments of 5 ml indicated by arrows First (I) second (II) and third (III) records obtained at intervals of 5 min Bladder filled until leakage of fluid (L) from the vesical cavity No interference with bladder tone is caused by the repetitive filling procedures (Cat 44)

The experimental situation thus provided still offered the opportunity to test sympathetic versus efferent parasympathetic influence on bladder tone

The cystometric curves showed identical effects in all the 4 animals The results are illustrated in Fig 6

(i) Firstly, the pressure volume curve of the intact bladder was determined (Fig 6 I) (ii) Next the afferent components of the sacral bladder innervation were interrupted on both sides The cystometrogram (Fig 6 II) now revealed a profound reduction of the spontaneous rhythmic activity loss of micturition reflex and furthermore a lower bladder tone as compared with the slope of the initial pressure volume diagram (iii) Bilateral section of the sympathetic chains was then performed leaving the efferent parasympathetic innervation alone intact Pressure adaptation to increasing stretch then became highly defective indicating a raised tonus particularly at the larger degrees of bladder filling (Fig 6 III) (iv) When finally also the parasympathetic efferent nerve supply was sectioned the slope of the cystometrogram again changed so as to reflect nearly the same stretch behavior as that of the intact bladder (Fig 6 IV)

3 *Repetitive cystometric examinations of the denervated bladder* With all extrinsic nerves bilaterally sectioned the resulting abolition of the micturition reflex implies the danger that the vesical wall might be unduely stretched unless special measures are taken against uncontrolled distension In the present experiments this requirement was fulfilled by interrupting the filling procedure at the first sign of fluid leakage from the external urethral orifice When performed in this way repetitive cystometric examinations did not affect the tone of the denervated bladder (Fig 7)

Discussion

It appears from the Introduction that opinions differ widely with respect to the influence of the vesical nerves on bladder tone despite the fact that essentially the same technical approach has been employed in previous investigations concerning this problem Thus clinical and experimental work has been based on intravesical pressure determinations by means of a closed recording system as introduced by Mosso and

Pellacani (1882) By the use of this method there is a considerable danger that the vesical wall might be unduly distended, whereby the stretch behavior is likely to be interfered with Langley and Whiteside (1951) and Tang and Ruch (1955) demonstrated such effect of overstretching the vesical wall in repetitive cystometric examinations A gradual reduction in vesical tone was shown subsequent to each filling procedure when performed in rapid succession and exceeding the micturition threshold From this observation the conclusion was drawn that the vesical "atony" following injuries to the spinal cord or section of the pelvic nerves is not a true atonic state, but a manifestation of bladder overdistension due to the abolition of the micturition reflex

In the present experiments, vesical tone was analysed from pressure-volume records obtained by cannulation of the bladder directly through the abdominal wall, whereas the urethral outlet was left intact (for further description of the method, see Murphy and Schoenberg 1960, Gjone and Setekleiv 1963 and Gjone 1965) With the open outlet, overdistension of the vesical wall could be avoided by interrupting the bladder filling at the micturition threshold or, in the absence of reflex micturition due to section of the vesical nerve supply, as soon as urethral leakage of fluid was noted The cystometrograms thus obtained, showed that repetitive bladder distension did not affect the slope of the pressure-volume records

The cystometric findings during a developing cystitis yield support to the view that bladder tone is closely dependent on the inherent properties of the vesical wall as particularly emphasized by Tang and Ruch (1955) Such dependency was further indicated by the striking similarity characterizing the slope of the pressure volume records from the bladder in the intact state and after section of its extrinsic nerve supply In agreement with numerous previous workers (see Introduction) the opinion is advanced that the tone of the bladder is primarily reflecting an autonomous funtion of the vesical wall This conclusion does not, however exclude the possibility that the vesical tone is modulated by a peripheral autonomic mechanism On the contrary a subordination to a tonic nervous influence is very likely since alterations in vesical tone have been produced in response to stimulation of numerous supraspinal structures (for references, see Langworthy and Kolb 1933, Peretti 1951 and Koikegami *et al* 1957)

The existence of a nervous mechanism influencing bladder tone through the parasympathetic and sympathetic vesical nerves was established by the cystometric findings during progressive autonomic denervation Subsequent to bilateral section of the parasympathetic nerve supply a reduction in vesical tone was demonstrated This effect yielded support to the traditional concept that the tone of the bladder depends mainly on excitatory influence from a sacral "center (see Introduction) Following additional sympathetic denervation the slope of the pressure-volume curve increased to the level of a nearly normal bladder tone Thus evidence was obtained that the relaxation following parasympathetic denervation could not be ascribed to an "atonic" state nor to bladder overdistension, but was the result of an inhibitory influence from the sympathetic vesical supply

Definite evidence of such antagonistic inhibitory effect appears from the cystometrograms obtained after primary sympathetic bladder denervation, associated with bilateral section of the afferent vesical fibres to the sacral cord Under influence of the efferent parasympathetic innervation alone the bladder now became markedly hypertonic The excitatory reaction, released by depriving the bladder of the sympathetic pathways disappeared following additional section of the remaining extrinsic nerve supply, *i e* by cutting also the efferent parasympathetic fibres

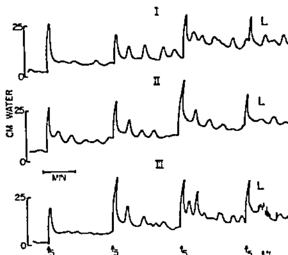


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3 *Repetitive cystometric examinations of the denervated bladder* With all extrinsic nerves bilaterally sectioned the resulting abolition of the micturition reflex implies the danger that the vesical wall might be unduely stretched, unless special measures are taken against uncontrolled distension In the present experiments this requirement was fulfilled by interrupting the filling procedure at the first sign of fluid leakage from the external urethral orifice When performed in this way, repetitive cystometric examinations did not affect the tone of the 'denervated' bladder (Fig 7)

Discussion

It appears from the Introduction that opinions differ widely with respect to the influence of the vesical nerves on bladder tone despite the fact that essentially the same technical approach has been employed in previous investigations concerning this problem Thus, clinical and experimental work has been based on intravesical pressure determinations by means of a 'closed' recording system, as introduced by Mosso and

Pellacani (1882) By the use of this method there is a considerable danger that the vesical wall might be unduly distended, whereby the stretch behavior is likely to be interfered with Langley and Whiteside (1951) and Tang and Ruch (1955) demonstrated such effect of overstretching the vesical wall in repetitive cystometric examinations A gradual reduction in vesical tone was shown subsequent to each filling procedure when performed in rapid succession and exceeding the micturition threshold From this observation the conclusion was drawn that the vesical "atony" following injuries to the spinal cord or section of the pelvic nerves is not a true atonic state, but a manifestation of bladder overdistension due to the abolition of the micturition reflex

In the present experiments vesical tone was analysed from pressure-volume records obtained by cannulation of the bladder directly through the abdominal wall, whereas the urethral outlet was left intact (for further description of the method, see Murphy and Schoenberg 1960, Gjone and Setekleiv 1963 and Gjone 1965) With the open

metrograms thus obtained, showed that repetitive bladder distension did not affect the slope of the pressure-volume records

The cystometric findings during a developing cystitis yield support to the view that bladder tone is closely dependent on the inherent properties of the vesical wall, as particularly emphasized by Tang and Ruch (1955) Such dependency was further indicated by the striking similarity characterizing the slope of the pressure volume records from the bladder in the intact state and after section of its extrinsic nerve supply In agreement with numerous previous workers (see Introduction) the opinion is advan

sympathetic and sympathetic vesical nerves was established by the cystometric findings during progressive autonomic denervation Subsequent to bilateral section of the para sympathetic nerve supply a reduction in vesical tone was demonstrated This effect yielded support to the traditional concept that the tone of the bladder depends mainly on excitatory influence from a sacral center (see Introduction) Following additional sympathetic denervation the slope of the pressure-volume curve increased to the

pathetic vesical supply

Thus, an excitatory parasympathetic and an inhibitory sympathetic influence previously shown to modulate the rhythmic vesical contractions (Gjone 1965) has been found to modulate the 'tone' of the bladder as well

References

CARPENTER, F G and W S ROOT Effects of parasympathetic denervation on feline bladder function *Amer J Physiol* 1951 *166* 686—691

DENNY BROWN D and E G ROBERTSON The state of the bladder and its sphincters in complete transverse lesions of the spinal cord and cauda equina *Brain* 1933 *56* 397—463

ELLIOTT T R, The innervation of the bladder and urethra *J Physiol* (Lond) 1907 *35* 367—445

EVANS J P Observations on the nerves of supply to the bladder and urethra of the cat with a study of their action potentials *J Physiol* (Lond) 1936 *86* 396—414

GJONE R Peripheral autonomic influence on the motility of the urinary bladder in the cat I Rhythmic contractions *Acta physiol scand* 1965 *65* 370—377

GJONE R and J SETEKLEIV Excitatory and inhibitory bladder responses to stimulation of the cerebral cortex in the cat *Acta physiol scand* 1963 *59* 337—348

HOLMES G Observations on the paralyzed bladder *Brain* 1933 *56* 383—396

KOIKEGAMI H T DODO Y MOCHIDA and H TAKAHASHI Stimulation experiments on the amygdaloid nuclear complex and related structures effects upon renal volume urinary secretion movements of urinary bladder blood pressure and respiratory movements. *Fol a psychiat neurol jap* 1957 *11* 157—206

KOLLBERG S and I PETERSÉN Cystometric studies of the effect of Lisidonil upon the bladder *Urol int* (*Basel*) 1963 *16* 46—64

LANGLEY L L and J A WHITESIDE Mechanism of accommodation and tone of urinary bladder *J Neurophysiol* 1951 *14* 147—152

LANGWORTHY O R and L C KOLB The encephalic control of tone in the musculature of the urinary bladder *Brain* 1933 *56* 371—382

LANGWORTHY O R L C KOLB and L G LEWIS *Physiology of micturition* Williams and Wilkins Co Baltimore 1940

MOSSO M A and P PELLACANI Sur les fonctions de la vessie *Arch ital Biol* 1882 *1* 92—108

MOSSO M A and P PELLACANI Sur les fonctions de la vessie *Arch ital Biol* 1882 *1* 291—324

MUNRO D The cord bladder — its definition treatment and prognosis when associated with spinal cord injuries *New Engl J Med* 1935 *215* 766—775

MURPHY J J and H W SCHOENBERG Physiology and surgery of the genitourinary system *Surg Clin N Amer* 1960 *40* 1615—1628

NESBIT R M and J LAPIDES Tonus of the bladder during spinal shock *Arch Surg* (*Chicago*) 1948 *56* 138—144

NESBIT R M W W VALK M SUTTER, R L BERRY R H LYONS K N CAMPBELL and G K MOE The effects of blockade of the autonomic ganglia on the urinary bladder in man *J Urol* (*Baltimore* 1947 *57* 242—250

PERETTI G Research on the postural tonus of the urinary bladder and on its neural regulation III Mechanism of action of the mesencephalic vesical center *Arch Fisiol* 1951 *51* (1) 41—45

PERETTI G Research on the postural tonus of the urinary bladder and on its neural regulation IV First observations on the influence of the cerebellum on the postural tonus of the vesical detrusor muscle *Arch Fisiol* 1951 *51* (1) 46—53

PRATHER, G C Urological aspects In Prather G C and F H Mayfield *Injuries of the spinal cord* C C Thomas Springfield Illinois 1953 52—194

RUCH T C Central control of the bladder *Handbook of physiology* Williams and Wilkins Co. Baltimore 1960 Sec 1 *II* 1207—1223

SHERRINGTON C S Postural activity of muscle and nerve *Brain* 1915 *38* 191—234

STEWART C C Mammalian smooth muscles — the cat s bladder *Amer J Physiol* 1900 *4* 185—208

TANG P.-C and T C RUCH Non neurogenic basis of bladder tonus *Amer J Physiol* 1955 *181* 249—257

Acta physiol scand 1966. 66 81—90

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Peripheral Autonomic Influence on the Motility of the Urinary Bladder in the Cat

III Micturition

By

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Abstract

The function of the urinary bladder serves a dual purpose, *viz*, the temporary storage and the periodical expulsion of urine During the storage phase the activity of the vesical wall is characterized by rhythmic contractions and by adaptation of the intramural tension in order to maintain a low intravesical pressure level ("tone") Both these changes in bladder motility are reflected by the pressure responses to a gradual increase of the intravesical volume (cystometrogram) The results of several recent investigations show that vesical storage function is primarily autonomous in nature (for references see

Gjone 1965 a, b) This conclusion is based upon the similar stretch behavior recorded by cystometric examination of the intact bladder and after section of its extrinsic nerve supply However, by partial, parasympathetic or sympathetic, bladder denervation evidence was provided, indicating that the rhythmic contractions as well as the adaptation of vesical "tone" were influenced by antagonistic autonomic effects (Gjone 1965a, b)

When a certain degree of bladder filling is reached, a critical tension develops in the bladder wall causing a steep and marked rise of the intravesical pressure level followed by the onset of micturition Thus, distension of the vesical wall up to an individual limit, the micturition threshold, appears to be an adequate stimulus for the elicitation of the micturition reflex At the same time, however, the initiation of micturition is primarily dependent on nervous regulation mediated through the autonomic supply to the vesical wall This clearly appears from the frequent observation by clinical workers that injuries to the pelvic nerves or to the corresponding level of the spinal cord are invariably followed by bladder dysfunction due to interference with the micturition reflex (For systematic reviews of the literature, see Fearnsides 1917, Learmonth 1931, Holmes 1933, Denny-Brown and Robertson 1933, Munro 1935, Langworthy, Kolb and Lewis 1940, Kuntz 1953, Prather 1953 and Plum 1962) From these clinical data general agreement was reached that the influence conducted by the parasympathetic vesical nerves is of paramount importance in the elicitation of a regular micturition act Definite evidence in support of such excitatory parasympathetic effects on micturition was provided in experimental studies, inasmuch as abolition of normal reflex micturition was always demonstrated subsequent to bilateral section of the pelvic nerves (for references see Elliott 1907, Barrington 1915, Denning 1926 Langworthy *et al* 1940, Mellanby and Pratt 1940 Kuntz and Saccomanno 1944, Ruch 1960 and Plum 1962)

Further experimental data concerning peripheral nervous regulation of micturition have been deduced from intravesical pressure changes caused by section and stimulation of the extrinsic vesical nerves As the traditional "closed" recording system was employed in all previous investigations, the influence of the different bladder nerves on the micturition act could not be directly demonstrated Intravesical pressure responses were however obtained, reflecting changes in vesical motility during the storage function These results were generally reported in terms of contraction or relaxation of the bladder wall, whereby an excitatory or inhibitory effect was indicated also with respect to the act of urine expulsion Thus, Elliott (1907) found that the parasympathetic nerves cause detrusor contraction and relaxation of the internal sphincter, whereas the sympathetic nerves mediate impulses resulting in detrusor relaxation and contraction of the internal sphincter This classical theory was later supported by Learmonth (1931), Holmes (1933), Mellanby and Pratt (1940) Prather (1953), Kollberg and Petersén (1963)

Detrusor contractions although smaller in amplitude and of shorter duration, have been obtained by stimulation also of the sympathetic vesical supply (MacDonald and McCrea 1930, Wang and Harrison 1939 Kuntz and Saccomanno 1944, Girado and Campbell 1959, Ingersoll Jones and Hegre 1961)

Other workers, also employing the closed recording technique, observed inhibitory effects on the detrusor contractions by stimulation of the parasympathetic as well as the sympathetic nerves to the bladder (Wang and Harrison 1939, Hegre and Ingersoll 1949, Edge 1955) Such inhibitory effects most constantly occurred by parasympathetic stimulation

Inhibitory effects on the micturition act have previously escaped direct observation due to the fact that bladder emptying was prohibited by the "closed" recording methods hitherto employed for studies of bladder motility In order to clarify the peripheral nervous regulation of micturition a technical modification is required, whereby inhibitory as well as excitatory effects can be demonstrated This requirement was fulfilled by the direct method of bladder cannulation described in preceding communications (Gjone and Setekleiv 1963, Gjone 1965a, b) Intravesical pressure recording was established through an ordinary cystostomy, whereas the bladder outlet was left untouched Because the emptying function of the bladder remained intact, a regular micturition act appeared in response to bladder distension The bladder contractions during micturition were reflected in the intravesical pressure record The parasympathetic and sympathetic influence on micturition was investigated by observing the effects following section and stimulation of each of these nerve supplies alternatively Since excitatory parasympathetic and inhibitory sympathetic effects have previously been shown to modulate the rhythmic activity and the tone of the bladder, similar nervous effects on micturition were searched for

In addition, the activity of the socalled internal sphincter was observed in the intact bladder during peripheral parasympathetic or sympathetic stimulation and after sectioning one or the other of these nerve supplies The smooth muscle activity in the sphincter region" was studied in each of these situations by cystoscopic examination and/or local pressure recording

Material and methods

previously (Gjone and Setekleiv 1963) In 3 cats a double-barrelled cystoscope was introduced through the cystostomy for combined intravesical inspection and pressure recording

The blood pressure in the femoral artery and the respiratory movements were recorded simultaneously in 12 and 10 animals respectively

The sympathetic and or the parasympathetic nerves to the bladder were then exposed by the operative technique described previously (Gjone 1965a)

monitoring oscilloscope

Results

The operative insertion of the recording catheter obviously caused some narrowing of the vesical cavity and moderate local traumatization of the bladder wall Yet micturition thresholds ranging from 10 to 70 ml were found after cannulation of the bladder, and by increasing the intravesical volume up to this individual limit a regular micturition act always ensued

By gradual filling of the bladder characteristic cystometrograms were obtained, terminating in a micturition curve ', illustrated in Fig 1 A The powerful bladder con-

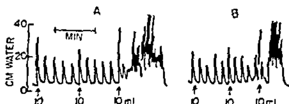


Fig 1 Complete voiding act initiated by volume increments of 10 ml at arrows A, before and B, after curarization (Cat 84)

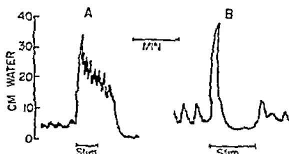


Fig 2 A Parasympathetic supply to the bladder interrupted by sectioning the sacral nerves bilaterally Intravesical volume 30 ml A regular micturition act was initiated by stimulating the peripheral end of the second sacral nerve (4 volts 0.32 mA 2 msec, and 50 cps) B Sympathetic chains also sectioned, intravesical volume 5 ml. Peripheral end of the sympathetic nerve trunk stimulated with 4 volts (0 32 mA), 5 msec, 30 cps No expulsion of fluid from the bladder during the initial pressure elevation (Cat 70)

tractions involved in the evacuation act are seen to build up from the spontaneous rhythmic activity which rapidly increases in frequency and amplitude Expulsion of fluid was initiated after a sudden rise in pressure, and from this moment on the voiding was accomplished with little reduction in the rate of flow until the bladder was empty

As previously shown (Gjone 1965b) a considerably lower micturition threshold ensued under chloralose-urethane than under nembutal anesthesia, whereas the micturition act itself was fulfilled in the same manner

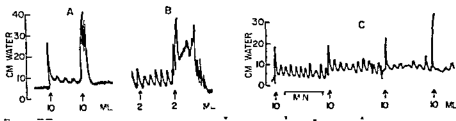
Neither changes in the micturition threshold nor in the "micturition curve" were observed after paralyzing the striated musculature by curarization (Fig 1)

Parasympathetic innervation

The peripheral parasympathetic fibres pass in the ventral sacral roots to reach the bladder through the pelvic nerves The latter also contain afferent nerve fibres from the vesical wall These join the sacral cord through the dorsal roots Thus, by isolating the ventral sacral roots from the dorsal roots, the efferent (parasympathetic) and the afferent sacral nerve supply can be interrupted and stimulated individually without interfering with the other component (Gjone 1965a)

A Section experiments

The effects of bilateral parasympathetic denervation of the bladder, without interfering with the sympathetic pathways were observed in 16 animals The influence of the efferent and afferent sacral innervation was studied separately by section of either the ventral nerve roots (6 expts) or the dorsal roots (4 expts Essentially the same results ensued by isolated efferent or afferent root sectioning as those following interruption of the spinal nerves From cystometrograms obtained immediately after such denerva-



tion the micturition reflex was found to be absent. At intravesical volumes exceeding the individual micturition thresholds a dribbling leakage of fluid occurred, closely resembling the "paradoxic incontinence" which is more appropriately characterized as overflow

B Effects of stimulation

Following sacral nerve sectioning the peripheral ends of the ventral roots were stimulated in 6 experiments, whereas the spinal nerves were stimulated in 12 animals In either case a regular micturition act was induced, as illustrated in Fig 2 A A sharp elevation of the intravesical pressure level is here seen to follow stimulation This initial pressure peak was found to appear after a short latency, as a rule not exceeding 2 sec After a further latency period of 2 to 5 sec expulsion of fluid was observed from the external urethral orifice Thereafter micturition continued despite termination of stimulation until evacuation of the bladder contents was complete

Micturition was produced by stimulating any of the three sacral nerves in the cat The most powerful effects were obtained from S_2, although complete evacuation of the bladder contents as a rule also resulted from stimulation of S_3 whereas S_1 yielded more inconstant responses For the elicitation of an ordinary micturition act intensities of 1 5—3 volts (0 12—0 24 mA) were required Frequency studies covering a range of 0 1—100 cps showed maximal effects at a rate of 40—60 cps at pulse lengths of 1—5 msec

Sympathetic nerves

In the cat the vesical supply from the sympathetic division of the autonomic system originates from Th_{13} and L_1—L_3 The nerves from each side, through which both efferent and afferent impulses are conveyed unite to form the hypogastric plexus, or presacral nerve, before entering the bladder wall No attempt was made to separate the efferent and afferent pathways when the sympathetic nerves were interrupted As described previously (Gjone 1965a) the sympathetic nerves were reached for sectioning and stimulation by bilateral dissection of the paravertebral chains just above the upper branch to the hypogastric plexus

1 Sympathetic denervation

In 18 animals the sympathetic supply was divided bilaterally under maintenance of an intact parasympathetic innervation A profound reduction of the micturition threshold constantly and momentarily occurred (Fig 3 A—B) This effect could in fact be used

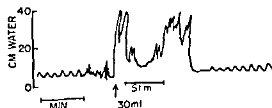


Fig 4 All extrinsic nerves to the bladder intact but sympathetic chains dissected free in the lumbar region Micturition threshold at 30 ml Voiding initiated by rapid fluid injection at arrow Interruption of micturition induced by stimulation of the intact sympathetic trunk (4 volts 4 msec and 30 cps) Micturition resumed and completed on termination of stimulation (Cat 68)

to verify that the proper nerves had been cut As a rule as little as 4—5 ml sufficed to initiate the micturition reflex Bladder emptying was then rapidly completed

B Stimulation experiments

Effects on the micturition act

The peripheral sympathetic supply to the bladder was left intact in these experiments (8 cats) because a preceding section of the same nerves was followed by such a marked shortening of the emptying phase that an appropriate background activity could not be maintained during stimulation A regular micturition act was induced by rapid filling of the bladder A few seconds after the initiation of micturition a stimulus of about 1 min duration was applied to the sympathetic trunks or to the presacral nerve As demonstrated in Fig 4 micturition was arrested during the entire period of stimulation The arrest was accompanied by a marked lowering of the intravesical pressure and by abolition of the rhythmic bladder contractions On termination of stimulation bladder evacuation was resumed and complete emptying was effected Such inhibitory stimulation effects closely resembled those previously obtained by cerebral stimulation (Gjone and Setekleiv 1963)

Effects on the storage phase

Stimulation of the peripheral ends of the cut sympathetic chains produced constant and marked effects on the rhythmic bladder activity as shown in Fig 2 B The regular vesical contractions were suspended during the entire period of stimulation and replaced by a diphasic response First there was an immediate and sharp rise of the intravesical pressure followed by a fall below the pre stimulatory level which was then gradually reached *The short lasting initial pressure elevation was never accompanied by any expulsion of fluid from the vesical cavity* It should further be noted that this effect always subsided quickly despite continued stimulation

The inhibitory response to sympathetic stimulation was studied under systematic frequency variations from 0 1—100 cps Maximal effects ensued within the range of 15—30 cps At these optimal parameters intensities of 2—4 volts (0 16—0 32 mA) were required to obtain significant responses

The denervated bladder"

In 12 of the experiments described above both the parasympathetic and the sympathetic nerve supplies were sectioned at termination of the experiment As it appears from the cystometrogram in Fig 3 C the micturition reflex was abolished When the level of overdistension was reached the characteristic dribbling leakage of fluid (overflow) was observed

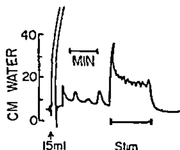


Fig 5 All extrinsic nerves interrupted Intravesical volume 15 ml Direct stimulation of the bladder wall through bipolar electrodes embedded in the trigonal region (5 volts 5 msec 50 cps) induced micturition Fluid evacuation ceased on termination of stimulation leaving a rest of 5 ml in bladder cavity (Cat 80)

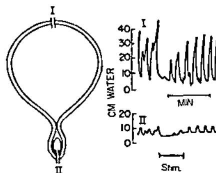


Fig 6 Simultaneous pressure recording from the vertex region (I) and the bladder neck (II) using a balloon at the latter site (further description in text) Intravesical volume 10 ml Both sympathetic chains cut Inhibition of muscular activity obtained in both regions by stimulation of the peripheral end of the sympathetic trunk (3 volts 5 msec 30 cps) (Cat 83)

Direct stimulation of the bladder wall was performed in 2 of these experiments through ball tipped electrodes fixed 8 mm apart to the dorsal surface near the base of the bladder (Fig 5) Evacuation of the vesical contents was constantly induced by stimuli (5 volts 5 msec 50 cps) applied to this site but contrary to the effects obtained from the para sympathetic nerves micturition immediately ceased on stopping the direct stimulation of the bladder musculature

The internal sphincter'

The internal urethral orifice is surrounded by crossing bundles of smooth muscle fibres They are continuous with the smooth muscle of the vesical wall although reinforced by increased thickness mainly of those bundles which are derived from the middle circular or oblique layers This more or less ring-shaped extension of vesical smooth muscle at the base of the bladder is generally referred to as the internal sphincter Furthermore functionally it has been considered largely independent of the bladder globe In view of the experimental data obtained in the present work particular problems thereby arise Therefore the functional role of the internal sphincter was made the subject of the following study

By cystoscopic examination during stimulation of the cut sympathetic chains the internal urethral orifice was seen to close slowly within a period of 5—10 sec No contraction of the detrusor muscle was seen to accompany this response The trigonal region on the other hand was clearly reacting to sympathetic stimulation by local contractions extending upwards beyond the ureteral orifices

Further elucidation of the closure function of the internal sphincter was aimed at



Fig 7 Muscular ring composing the internal sphincter divided by vertical retropubic incision (further description in text) Micturition threshold unchanged (30 ml) *A* Prolonged incomplete micturition act after step-wise volume increase by 10 ml at arrows *B* Interruption of micturition induced by stimulation of the sympathetic nerve trunk (4 volts 5 msec 20 cps) (Cat 90)

through simultaneous recording of the sympathetic stimulation effects on the intraluminary pressure in the proximal part of the urethra and in the bladder cavity, respectively (2 cats) For this purpose a ballon was used at the former site The results obtained appear from Fig 6 The two records are seen to reflect essentially the same pressure response to stimulation indicating a relaxation also of the socalled sphincter muscle

Additional evidence in support of the view that closure of the bladder outlet is effected by relaxation and not by contraction, was obtained by dividing the muscular tissue surrounding the internal orifice and the proximal part of the urethra (2 cats) A vertical retropubic incision of 15 mm length was made, whereby the serosal and muscular layers of the bladder neck were split leaving only the mucous membrane intact The urinary bladder remained continent subsequent to this procedure, as appears from the cystometric examination illustrated in Fig 7 A Furthermore interruption of micturition occurred in response to stimulation of the sympathetic chains (Fig 7 B) With respect to the emptying function of this bladder a significant impairment was noted, inasmuch as a prolonged and incomplete voiding act ensued

Discussion

In accordance with the reports of numerous earlier workers (see introductory part, the *parasympathetic* bladder supply was found by sectioning and stimulation to exert excitatory effects on micturition As mentioned in the introduction some earlier workers advanced the opinion that the parasympathetic nerves are also conducting inhibitory effects on micturition No evidence was provided in support of this concept

With respect to the initiation of micturition it is of particular interest that the reflex is completely abolished when the denervation is confined to the sacral afferent fibres In other words the efferent parasympathetic pathway does not convey impulses exciting the bladder emptying mechanism unless linked together with afferent signals from the vesical wall It seems near at hand to correlate these observations to the electric impulses which have been recorded in afferent nerves from the bladder to the sacral cord (Talaat 1937 Iggo 1955 and Plum 1962) Bursts of impulses were found to accompany the rising phase of each rhythmic intravesical pressure variation, whereafter the rate of firing momentarily decreased A minimal impulse activity was then recorded during the whole interval preceding the subsequent wave of contraction

A dual autonomic influence on the micturition act was established by the fact that inhibitory effects were shown to be mediated by the sympathetic pathway to the bladder Firstly, such inhibitory influence was indicated by the profound reduction of the micturition threshold following sympathetic denervation This finding has been reported also by earlier investigators as Learmonth (1931), Bucy, Huggins and Buchanan (1937) and Kuntz and Saccomanno (1944) Definite evidence of the inhibitory

character of the sympathetic influence was provided by the induction of "micturition arrest" in response to stimulation of the intact sympathetic nerve trunks. This response has to our knowledge not been observed previously. By stimulation of the peripheral cut end of the sympathetic chain a diphasic effect on the intravesical pressure was noted, closely resembling the response reported by previous workers using the traditional recording method (Langworthy *et al* 1940, Kuntz and Saccomanno 1944, Hegre and Ingersoll 1949, Edge 1955). Based on the pressure peak, initiating this diphasic response, the view was widely adopted (see Introduction) that excitatory as well as inhibitory effects on the micturition reflex are conducted through the sympathetic pathway. Due to the modified recording technique employed in the present experiments it could be shown, however, that the initial pressure peak is not accompanied by any expulsion of fluid from the vesical cavity. Hence, this change in the intravesical pressure cannot be attributed to an excitation of the micturition reflex. According to the cystoscopic examination, it is most likely due to contraction of the trigonal muscle and the distal part of the ureters, as has previously been observed by Learmonth (1931) and Langworthy *et al* (1940).

The internal sphincter. The dual autonomic influence on the micturition act does not imply the acceptance of the traditional subdivision of the bladder in two separate functional components, *i e*, "detrusor" and the "internal sphincter". From an anatomical point of view the separation of the bladder globe from a sphincter muscle is merely theoretical as already stated in 1883 by F. Le Gros Clark. Based on meticulous histological studies he concluded that there is no evidence of such boundaries at the base of the bladder. The same concept has been advanced by recent workers (Clegg 1957, Allert and Buscher 1964), who found that the vesical smooth muscle could be traced as a continuum down below the neck of the urinary bladder. In accordance with these anatomical data, the opening of the bladder outlet during micturition was explained by Lapides (1958) and Plum (1962) as the result of a shortening and widening of the urethra effected by contraction of the smooth muscle at the base of the bladder. It was assumed that the outlet was closed by an elongation of the urethra due to muscular relaxation. Thus the socalled "internal sphincter" muscle was supposed to act in phase with and as part of the detrusor muscle contracting and relaxing simultaneously with this. This theory is strongly supported by observations made in the present experiments: (i) Complete evacuation of the vesical contents resulted from parasympathetic stimulation alone, (ii) bladder relaxation resulting from sympathetic stimulation was associated with pressure reduction also in the outlet region, (iii) no contraction was seen in the socalled sphincter musculature by cystoscopic examination in the latter experiment, (iv) the bladder remained continent and interruption of micturition was obtained in response to sympathetic stimulation after division of the "internal sphincter".

References

ALLERT, M. L. and H. K. BUSCHER: Zur Diagnose und Therapie der akuten und chronischen Harnverhaltung bei Erkrankungen des Nervensystems. *Dtsch. Z. Nervenheilk.* 1964. *186.* 395—409.

BARRINGTON, F. J. F.: The nervous mechanism of micturition. *Quart. J. exp. Physiol.* 1915. *8.* 33—71.

BOYCE, P. C., C. HUGGINS and D. N. BUCHANAN: Sympathetic innervation of external sphincter of human bladder. *Amer. J. Dis. Child.* 1937. *54.* 1012—1018.

CLEGG, E. J.: The musculature of the human prostatic urethra. *J. Anat. (Lond.)* 1957. *91.* 345—351.

DENNING, H Untersuchungen uber die Innervation der Harnblase und des Mastdarmes *Z Biol* 1924 *80* 239—254

DENNY BROWN D and E. G ROBERTSON, On the physiology of micturition *Brain* 1933 *56* 149—190

DENNY BROWN, D and F G ROBERTSON, The state of the bladder and its sphincters in complete transverse lesions of the spinal cord and cauda equina *Brain* 1933 *56* 397—463

EDGE, N D, A contribution to the innervation of the urinary bladder of the cat *J Physiol (Lond)* 1955 *127* 54—58

ELLIOTT T. R., The innervation of the bladder and urethra *J Physiol (Lond)* 1907 *35* 367—445

EVANS J P Observations on the nerves of supply to the bladder and urethra of the cat, with a study of their action potentials *J Physiol (Lond)* 1936 *86* 396—414

FEARNSIDES E. G The innervation of the bladder and urethra, a review *Brain* 1917 *15* 149—187

GIRADO J M and J B CAMPBELL, The innervation of the urethra of the female cats *Exp Neurol* 1959 *1* 44—64

GJONE R, Peripheral autonomic influence on the motility of the urinary bladder in the cat. I Rhythmic contractions *Acta physiol scand 1965a 65* 370—377

GJONE R, Peripheral autonomic influence on the motility of the urinary bladder in the cat. II Tone *Acta physiol scand* 1966 *66* 72—80

GJONE R. and J SETEKLEIV Excitatory and inhibitory bladder responses to stimulation of the cerebral cortex in the cat *Acta physiol scand 1963 59* 337—348

HEGRE, E S and E. H INGERSOLL, An analysis of regional variations in the response of the detrusor muscle to electrical stimulation of the hypogastric nerves *J Urol (Baltimore)* 1949 *61* 1037—1047

HOLMES G, Observations on the paralyzed bladder *Brain* 1933 *56* 383—396

IGGO A. Tension receptors in the stomach and the urinary bladder *J Physiol (Lond)* 1955 *128* 593—607

INGERSOLL E H L. L JONES and E S HEGRE Role of the sympathetic nervous system in urinary bladder function in the cat *Neurology* 1961 *11* 46—55

KOLLBERG S and I PETERSÉN Cystometric studies of the effect of Lisidonil upon the bladder *Urol int (Basel)* 1963 *16* 46—64

KUNTZ A The urinary bladder *The autonomic nervous system* Lea & Febiger Philadelphia 1953 277—288

KUNTZ A and G SACCOMANNO Sympathetic innervation of the detrusor muscle *J Urol (Baltimore)* 1944 *51* 535 542

LANGWORTHY O R L. C KOLB and L G LEWIS *Physiology of Micturition* Williams and Wilkins Co Baltimore 1940

LAPIDES J Structure and function of the internal vesical sphincter *J Urol (Baltimore)* 1958 *80* 341—319

LEARMONTH J R A contribution to the neurophysiology of the urinary bladder in man. *Brain* 1931 *54* 147—176

LE GROS CLARK F Some remarks on the anatomy and physiology of the urinary bladder and of the sphincters of the rectum *J Anat Phys* 1883 *17* 442—459

MACDONALD A D and E D MCCREA Observations on the control of the bladder — The effects of nervous stimulation and of drugs. *Quart J exp Physiol* 1930 *20* 379—391

MELLANBY J and L G PRATT The reactions of the urinary bladder of the cat under conditions of constant volume *Proc roy Soc Med* 1940 Series B *128* 186—201

MUNRO D The cord bladder — its definition treatment and prognosis when associated with spinal cord injuries *New Engl J Med* 1935 *215* 766—774

PLUM F Bladder dysfunction In D Williams, *Modern trends in neurology* Butterworths London 1962 151—172

PRATHER G C Urological aspects In Prather G C and F H Mayfeld *Injuries of the spinal cord* C C Thomas Springfield Illinois 1953 52—194

RUCH T C Central control of the bladder *Handbook of physiology* Williams and Wilkins Co Baltimore 1960 Sec 1 II 1207—1223

TALAAT M., Afferent impulses in the nerves supplying the urinary bladder *J Physiol (Lond)* 1937 89 1—13

WANG S C and F HARRISON The nature of the bladder responses following stimulation of the anterior hypothalamus *Amer J Physiol* 1939 *125* 301—309

Acta physiol scand 1966 66 91—102

From the Neurophysiological Laboratory at the Anatomical Institute University of Oslo Norway

Excitatory and Inhibitory Bladder Responses to Stimulation of 'Limbic', Diencephalic and Mesencephalic Structures in the Cat

By

RAGNAR GJONE

Received 28 April 1965

Abstract

Gjone R *Excitatory and inhibitory bladder responses to stimulation of limbic diencephalic and mesencephalic structures in the cat* Acta physiol scand 1966 *66* 91—102 — By means of intravesical

findings are reported Within the amygdaloid nuclear complex and the pyriform cortex excitatory bladder responses were obtained on stimulation of the basolateral division and inhibition on stimulation of the corticomedial area By hypothalamic stimulation a dorsolateral excitatory and a ventromedial inhibitory field was found Inhibitory bladder responses were demonstrated from the posterior cingulate gyrus and from the reticular formation of the upper brain stem Predominantly excitatory responses were produced by stimulation of the superior colliculi and from the periaquaeductal grey substance Excitatory responses from all these regions were abolished by section of the parasympathetic bladder supply but remained unaltered by sympathetic denervation Inhibitory responses were abolished when the sympathetic bladder supply was cut Thus excitatory effects of stimulation were identified as parasympathetic and inhibitory effects as sympathetic in nature

Supraspinal influence on vesical function was at first demonstrated by operative exposure of the urinary bladder Thus contractions of the bladder the intestinal canal and the uterus were seen in response to mechanical or electrical irritation of the medulla oblongata (for references see Schonberg 1864) In 1876 Bochefontaine demonstrated contractions of the exposed bladder by faradic stimulation of the pericruciate cortex somatic sensory motor area I in dogs

Most later data were obtained by intravesical pressure recording Since alterations in the mural tension are mediated to the fluid contents of the bladder excitatory or inhibitory effects on vesical motility are reflected by a rise or a fall in the fluid pressure

respectively Systematic pressure volume determinations were obtained from the urinary bladder by means of a permanent catheter, inserted through the urethra or directly through the vesical wall In either case the bladder outlet was occluded if necessary by a ligature, whereby a 'closed recording system was established Bladder excitation was thereby manifested through a pressure elevation indicating the elicitation of powerful detrusor' contractions like those involved in the micturition act, whereas inhibitory effects of supraspinal stimulation were represented by a lowering of the intravesical pressure (tone) and by a reduction or abolition of the rhythmic vesical activity i e by motility changes during the vesical storage phase only This technical approach has remained essentially the same since the end of the past century

The storage activities (Gjone 1965 a, b) as well as the micturition act (Gjone 1965 c) were shown to be modulated through excitatory parasympathetic and inhibitory sympathetic influence It should be noted however, that the reservoir activities are primarily autonomous in character, and only to a minor extent influenced from the nervous system The micturition reflex on the other hand is primarily dependent on nervous regulation (Gjone 1965 c) With respect to previous experimental data concerning supraspinal influence on the urinary bladder it seems likely that inhibitory effects of stimulation were liable to escape observation inasmuch as only excitatory effects on the micturition reflex were indicated by the traditional recording technique In accordance with these considerations predominantly excitatory areas have previously been identified at the encephalic level whereas bladder inhibition only occasionally has been reported

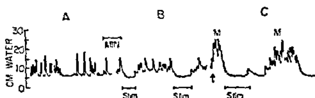
In a preceding investigation (Gjone and Setekleiv 1963) bladder responses to stimulation of the cerebral cortex were studied by intravesical pressure recording without interfering with the vesical emptying function By this modification of the recording method excitatory effects were represented by the elicitation of a regular micturition act whereas inhibition was indicated by the interruption of micturition The topographical distribution of cortical points yielding excitatory or inhibitory bladder responses to electrical stimulation was thereby further clarified Five zones were outlined from which the following patterns of bladder influence ensued

In agreement with previous observations both excitatory and inhibitory bladder responses were elicited from the somatic sensory motor area I In addition similar effects were obtained from the somatic sensory motor area II (the anterior ectosylvian and the anterior sylvian gyri) Further, exclusively excitatory responses were induced from the supracallosal part of the anterior cingulate gyrus whereas inhibitory bladder responses only could be produced from the subcallosal part of the cingulate cortex and the orbital gyrus

The present work represents a continuation of the preceding investigation concerning bladder responses to stimulation of supraspinal areas By stereotaxic stimulation of limbic diencephalic and mesencephalic structures points yielding excitation or inhibition of the micturition reflex were systematically explored by means of the modified recording technique which leaves the vesical emptying function intact In addition the role of the peripheral parasympathetic and sympathetic bladder innervation in mediating the centrally evoked effects were studied in nerve sectioning experiment

Material and methods

38 cats of both sexes (weight 1 9—3 7 kg were used They were anesthetized by i p injections of pentobarbital sodium (Nembutal Abbot) As a rule one single dose of 40 mg kg b w was sufficient to perform the operations required as well as to secure a relatively steady anesthet c



bladder with 30 ml at arrow (Cat 2)

level during the first hours of stimulation Additional doses of 6 mg kg nembutal were given if necessary according to the criteria mentioned elsewhere (Gjone 1965 a)

Records of the intravesical pressure respiratory movements (25 cats) and blood pressure (23 cats) were obtained on a Model 5 C Grass polygraph by the methods previously described (Gjone and Setekleiv 1963)

section being stained according to the Nissl method

A square wave stimulator with a monitoring oscilloscope for output control was used Pulse durations of 0 1—10 msec were employed at stimulus intensities ranging from 0.5 to 10 volts (about 0 04 to 0.8 mA) The frequency varied from 1 to 100 cps A pulse duration of 2 msec and a frequency of 50 cps were most commonly used With these parameters an intensity of 2—4 volts were in most instances found to be just above the threshold value A minimum interval of 3 min was allowed between each stimulus

Bilateral section of the sympathetic or parasympathetic nerves to the bladder was performed (as described previously (Gjone 1965 a

Results

In the following *excitatory* effects of cerebral stimulation refer to the elicitation of a regular micturition act *inhibition* refers to interruption of reflex micturition initiated by rapid bladder filling Fig 1 illustrates the greater significance which may be attributed to the latter response as compared with the reduction in rhythmic activity and vesical 'tone' recorded by the 'closed method' previously employed The decrease in rhythmic activity and "tone" of the bladder resulting from cerebral stimulation (B) is almost indistinguishable from the spontaneously occurring cessations of the pressure fluctuations (A) In C interruption of micturition is produced by stimulation of the same cerebral electrode site as in B

A The cerebral cortex

Apart from the 5 responsive cortical zones previously outlined (Gjone and Setekleiv 1963 effects on the micturition reflex were produced from two additional areas within

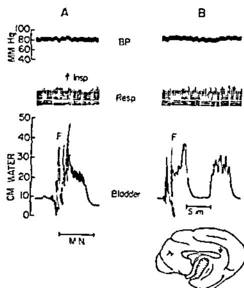


Fig 2 *A* Regular micturition act initiated by rapid bladder filling with 25 ml at F *B* Micturition initiated in the same way at F Stimulation (4 volts 0 32 mA 40 cps 2 msec) of the posterior cingulate area (at arrow on inset diagram) Interruption of micturition No effects on blood pressure and respiratory movements (Cat 42)

the 'limbic' cortex of the medial and ventral surfaces of the hemisphere, *viz*, the posterior cingulate region and the pyriform area

The posterior cingulate region was explored in 4 cats In 3 of these animals inhibitory effects were consistently obtained by stimulation of a small area closely surrounding the responsive point indicated on the inset diagram in Fig 2 B

The pyriform cortex was stimulated in 11 animals Of a total of about 50 electrode sites in this region 1 3 yielded micturition upon stimulation 1/3 inhibited micturition and the remaining electrode placements in the surroundings of the hatched areas in Fig 3 A gave no significant effect Excitatory responses were predominantly obtained from the posterolateral part of this area, whereas the inhibitory points were located in its anteromedial part

This separation of an excitatory from an inhibitory field, as determined by stereotaxic stimulation (Fig 4) was in 3 animals confirmed by direct stimulation of the exposed cortical surface Excitatory as well as inhibitory effects on micturition were in these experiments repeatedly produced in one and the same animal by changing the site of stimulation (Fig 3) The cortical origin of the responses was confirmed by their disappearance after local application of 1 per cent lidocaine (Xylocain® Astra) to the pyriform cortex

B The amygdaloid nuclear complex and adjacent structures

Bladder responses to stimulation of the amygdaloid nuclear complex and its surroundings were studied in 19 cats About 270 different loci were stimulated The following topographical organization was found (Fig 4)

Excitation (induction of micturition) was obtained from 68 points within the amygdaloid region and adjacent structures In the anterior part of the amygdaloid complex (A and B) there was some overlap with points yielding inhibitory effects Within the more well-defined amygdaloid nuclear groups bladder excitation was predominantly

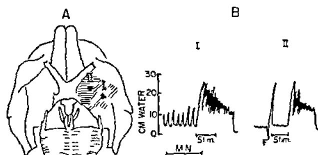


Fig 3 *A* Ventral aspect of the hemisphere illustrating area from which bladder responses

elicited from the lateral nucleus and from part of the magnocellular division of the basal nucleus (C—F)

Inhibition (micturition arrest) was induced from 60 needle positions in the amygdaloid region and its surroundings Apart from inhibitory points located in the anterior amygdaloid area, such points were almost exclusively confined to the corticomedial and central nuclei and the parvocellular division of the basal nucleus

In order not to overlook any responsive loci stimulus intensities of 4—5 volts were used, the stimulus threshold for both types of bladder responses being about 2—3 volts

Bladder responses were obtained from 5 of the 35 points stimulated within the claustrum, pallidum and putamen All of these 5 points were situated within the putamen (Fig 4 C—E) mostly at the border to surrounding responsive areas It seems likely that these effects were due to spread of the current to the adjacent amygdaloid nuclear complex

Micturition occasionally followed stimulation of the lateral part of the adjacent internal capsule (Fig 4) On the other hand, interruption of micturition predominated in its medial portion

The hippocampus No significant effects were obtained by stimulation of the temporal end of the hippocampus

C The diencephalon

The thalamus Micturition was elicited by stimulation of scattered points within the midline nuclei (Fig 5), whereas no significant effects were produced from the other thalamic nuclei

The hypothalamus Conspicuous effects on micturition were elicited from the hypothalamus at low stimulus strengths The threshold values seldom exceeded 1—1 5 volts (0 08—0 12 mA) When intensities of more than 4 volts were employed, inhibitory

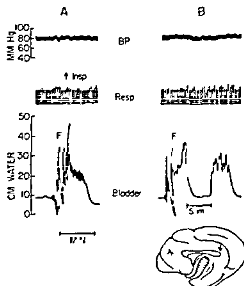


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Fig 5 Frontal sections in rostro caudal direction (*A—D*) through the dien cephalon of the cat illus trating bladder responses to stimulation of the pre optic and septal regions thalamus and hypothala mus Symbols and abbreviations as in Fig 4

Excitation was predominantly elicited from the preoptic and lateral supraoptic regions as well as from the dorsolateral part of the tuberal region and the posterior hypothalamus Some points extended into the lateral septal region (Fig 5 A) Inhibition occurred mainly in response to stimulation of the ventromedial areas within the tuberal region However there was a considerable intermingling with points yielding the opposite effect

D The mesencephalon

Mesencephalic structures were explored in 6 animals A total of 65 points were stimulated (Fig 6) Bladder excitation was obtained from the central grey substance, from the posterior part of the superior colliculi and the posterior commissure, whereas inhibitory effects were encountered by stimulation of points within the re icular substance and the anterior part of the superior colliculi

SN substantia nigra
SO n supraopticus
Spt area septalis
THP tractus habenulo-peduncularis
TO tractus opticus
TTC tractus tegmentalis centralis
VA n ventralis anterior
VL n ventralis lateralis
VM n ventralis medialis
VPL n. ventralis postero-lateralis
VPM n ventralis postero-medialis

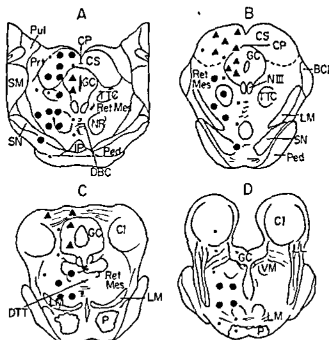


Fig 6 Frontal sections in rostro-caudal direction (*A—D*) through the mesencephalon of the cat showing the distribution of points yielding excitatory or inhibitory effects on urinary bladder motility Symbols and abbreviations as in Fig 4

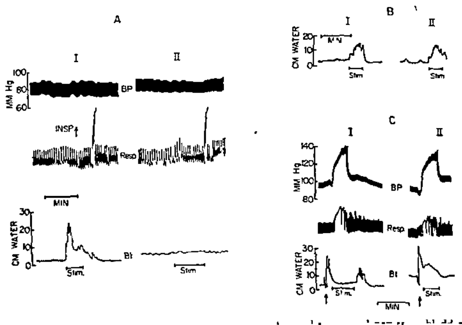
All responses from the mesencephalic level were characterized by low stimulus thresholds Thus, significant effects were produced at intensities of about 1 volt (0 08 mA) In mapping the responsive loci a stimulus intensity of 2 volts was ordinarily used

Concomitant changes in blood pressure and respiratory movements

With the exception of the posterior cingulate gyrus the effects on the bladder were frequently associated with alterations in blood pressure and respiration Inhibitory bladder responses produced from the hypothalamus and from the mesencephalic reticular substance were constantly accompanied by a marked rise in blood pressure as well as by conspicuous changes in the respiratory movements Slowing associated with reduction of the respiratory excursions or complete arrest in inspiration, was characteristic of this response From the other brain structures, influencing the urinary bladder, no definite and constant pattern of blood pressure and respiratory effects was demonstrated

Peripheral pathways mediating the supraspinal effects on bladder motility

Attempts to determine the peripheral pathways conveying the central effects were made in 12 animals At the end of the experiment bilateral section of the parasympathetic or sympathetic vesical supply was performed with the electrode fixed in a position giving excitation or inhibition of the micturition reflex The study of the changes thereby caused in the bladder effects was directed towards responsive points within the sensory motor cortical areas I and II as well as towards the orbital gyrus, the pyriform cortex the posterior part of the cingulate gyrus the amygdala the hypothalamus, the periaqueductal grey and towards the reticular substance of the mesencephalon By parasym



bilateral section of the parasympathetic nerves to the bladder (spinal nerves L_7—S_3) the
response is completely abolished (II) (Cat 59)

piratory movements remained unaltered (Cat 74)

pathetic or sympathetic bladder denervation the excitatory and inhibitory responses to supraspinal stimulation were influenced as seen from the records in Fig 7 (no matter the localization of the excitatory or inhibitory points, respectively).

In Fig 7 A excitatory effects (I) induced by stimulation of the somatic sensory-motor area I were abolished (II) after bilateral section of the parasympathetic bladder supply (sacral nerves) In Fig 7 B micturition (I) induced by stimulation of the basolateral part of the amygdala persisted after bilateral section of the sympathetic bladder nerves (II)

In Fig 7 C the interruption of micturition (I) evoked from the medial hypothalamus was abolished by bilateral section of the sympathetic vesical supply, whereas the accompanying effects on the blood pressure and the respiratory movements remained unaltered (II)

It should be noted that the results of sectioning the parasympathetic nerve supply on supraspinal inhibitory effects could not be determined in these acute experiments because the micturition reflex is always abolished by parasympathetic denervation

Discussion

Excitatory and inhibitory bladder responses to supraspinal stimulation were in the present work demonstrated by induction or interruption of the micturition act, respectively The parasympathetic bladder nerves were identified as the peripheral pathway mediating excitatory effects whereas the inhibitory influence was shown to be conveyed through the sympathetic nerves No conclusive evidence of such dual autonomic regulation from the supraspinal level has been obtained by previous workers (see Ingersoll, Jones and Hegre 1961)

Bladder responses have earlier been elicited from all the brain areas reported here to influence micturition However inhibitory effects of cerebral stimulation have seldom been described (see below) Excitatory responses have been found to predominate in areas from which bladder inhibition only could be demonstrated in the present experiments This discrepancy is likely due to differences in the recording methods Thus a diphasic alteration in the intravesical pressure, a short elevation followed by inhibition, has been interpreted as an excitatory effect when the traditional "closed recording system" has been used By the open outlet method it has been shown that this intravesical pressure elevation when elicited by peripheral sympathetic stimulation is not accompanied by expulsion of fluid from the bladder By cystoscopy this sharp initial pressure peak is seen to be associated with a contraction of the trigonal muscle, whereas the response of the bladder as a whole is predominated by relaxation as also indicated by the longlasting second phase of the intravesical pressure alteration Thus a sharp pressure peak obtained by intravesical pressure recording is not considered indicative of an excitatory bladder response On the contrary it may just as well represent the initial phase of an inhibitory response The true nature of such effects can only be determined by the use of the open outlet method Thereby an explanation is offered to the fact that a different functional organization ensued in the present study as compared to previous reports

The posterior cingulate gyrus In the present study inhibition of micturition was exclusively obtained whereas Kremer (1947) described the opposite pattern of bladder response *viz* contraction of the vesical wall Excitation as well as inhibition were reported in response to stimulation of the same region by Hennemann (1948) and Smith (1949) as judged by the pressure records from the closed vesical cavity

The pyriform cortex From the present results this region may be subdivided into a posterolateral area yielding predominantly excitatory responses, and an anteromedial area from which exclusively inhibitory effects were produced These data grossly correspond to previous findings Thus Uvnas (1947), Hennemann (1948) Smith (1949) and Kaada (1951) produced micturition mainly from the posterior part of this area, and inhibition of the rhythmic activity and tone of the bladder has been obtained by stimulation of its anterior part (Koikegami *et al* 1957)

The amygdaloid nuclear complex The distribution of the responsive points allows a clear subdivision of this region into a basolateral excitatory zone and a corticomedial inhibitory zone with respect to its effect on micturition In several previous studies micturition has been reported to occur incidentally in response to stimulation of various parts of the amygdaloid complex Thus Ursin and Kaada (1960 [illegible] mainly from the lateral amygdaloid area, an [illegible] present findings On the other hand, Magnus or [illegible] tion is produced predominantly from the medial [illegible] is workers concluded that there is no evid[illegible] gdaloid

nuclear complex with respect to the effect on bladder motility (McLean and Delgado 1953, Shealy and Peele 1957, Gloor 1960) By the use of a continuous pressure recording from the closed vesical cavity Koikegami *et al* (1957) demonstrated both excitatory and inhibitory bladder responses to amygdaloid stimulation From the distribution of the responsive loci a subdivision of the nuclear complex was made into a mediobasal excitatory region and a central inhibitory region These findings are seen to be in part confirmed in the present experiments

Apart from the effects on the urinary bladder the amygdaloid nuclear complex is known to influence other autonomic functions and various patterns of behavior Particular interest is attached to the findings by Ursin and Kaada (1960) In animal studies they separated a basolateral zone, causing "fear" (flight), and a corticomedial zone, giving aggressive reactions The fact that structures causing excitation or inhibition of micturition so closely correspond to the amygdaloid origin of fear and anger, respectively, may be of relevance to the common experience that micturition does not take place in the state of anger, whereas fear is well known to be accompanied by an increase of the urge to micturate

The hypothalamus From the present experiments it seems justified to distinguish a dorsolateral excitatory from a ventromedial inhibitory field, with respect to micturition, although the two areas are not sharply outlined It is of particular interest that the basolateral excitatory amygdaloid area recently has been shown to project to the dorsolateral hypothalamic nuclei whereas the inhibitory corticomedial amygdaloid nuclear group has been found to project to the ventromedial hypothalamic area (Nauta 1961) The present work thus provides physiological data in support of a corresponding functional differentiation

Bladder effects from the hypothalamus are usually associated with a variety of other autonomic phenomena The present results of hypothalamic stimulation coincide in part with the evidence previously obtained in favour of an anterior excitatory (parasympathetic) area and a posterior inhibitory (sympathetic) region, as suggested by Kabat Magoun and Ranson 1936, Wang and Harrison 1939, Crossman and Wang 1955, Ranson Kabat and Magoun 1956 Gellhorn 1959

The mesencephalon In accordance with the results obtained by Henneman (1948) and Smith (1949) inhibitory bladder responses were produced by stimulating the mesencephalic reticular substance In addition exclusively inhibitory effects on micturition were evoked from the anterior part of the superior colliculus Excitatory responses only were obtained by stimulation of the posterior part of the superior colliculus, the posterior commissure and the periaqueductal grey This excitatory area was indicated by Tang (1955) through the results of brain stem transections, but in those experiments the exact localization of the excitatory points could not be established

The peripheral pathways mediating the effects of supraspinal influence

Previously excitatory parasympathetic and inhibitory sympathetic bladder effects have been established (Gjone 1965 a b c) An intact peripheral autonomic nerve supply is required in order to maintain the regular reflex micturition whereas the storage activities *viz* the spontaneous rhythmic activity and the tone of the bladder are to a considerable extent independent on extrinsic nervous regulation In the present study the parasympathetic pathway has been found to mediate the excitatory influence on the micturition reflex from various supraspinal levels whereas the sympathetic bladder nerves were identified as the pathway for the inhibitory cerebral influence

References

BOCHEFONTAINE, L T, Étude expérimentale de l influence exercee par la faradisation de l écorce grise du cerveau sur quelques fonctions de la vie organique *Arch Physiol norm path* 1876 *8* (Sér 2) 140—172

GJONE R Peripheral autonomic influence on the motility of the urinary bladder in the cat I Rhythmic contractions *Acta physiol scand* 1965a *65* 370—377

GJONE R, Peripheral autonomic influence on the motility of the urinary bladder in the cat II Tone' *Acta physiol scand* 1966 *66* 72—80

GJONE R Peripheral autonomic influence on the motility of the urinary bladder in the cat III Micturition *Acta physiol scand* 1966 *66* 81—90

GJONE R and J SETEKLEIV Excitatory and inhibitory bladder responses to stimulation of the cerebral cortex in the cat *Acta physiol scand* 1963 *59* 337—348

GLOOR P Amygdala *Handbook of physiology* Williams and Wilkins Co Baltimore 1960 Sec 1, II 1395—1420

GROSSMAN R G and S C WANG Diencephalic mechanism of control of the urinary bladder of the cat *Yale J Biol Med* 1955—1956 *28* (3—4) 285—297

HENNEMAN E The central control of the bladder *Trans Amer neurol Ass* 1948 *73* 150—153

INGERSOLL E H L I JONES and F S HEGRE Role of the sympathetic nervous system in urinary bladder function in the cat *Neurology* 1961 *11* 46—55

JASPER H H and C AJMONE MARSAN *A stereotaxic atlas of the diencephalon of the cat* National Research Council of Canada Ottawa 1954

KABAT H H W MAGOUN and S W RANSON Reaction of the bladder to stimulation of points in the forebrain and midbrain *J comp Neurol* 1936 *63* 211—239

KOIKEGAMI H T DODO Y MOCHIDA and H TAKAHASHI Stimulation experiments on the amygdaloid nuclear complex and related structures effects upon renal volume urinary secretion movement of urinary bladder blood pressure and respiratory movements *Folia psychiat neurol jap* 1957 *11* 157—206

KREMER W F Autonomic and somatic reactions induced by stimulation of the cingular gyrus in dogs *J Neurophysiol* 1947 *10* 371—379

KAADA B R Somato-motor autonomic and electrocorticographic responses to electrical stimulation of rhinencephalic and other structures in primates cat and dog *Acta physiol scand* 1951 *24* (Suppl 83)

MAGNUS O and H J LAMMERS The amygdaloid nuclear complex *Folia psychiat neerl* 1956 *59* 555—582

MC LEAN P D and J M R DELGADO Electrical and chemical stimulation of frontotemporal portion of limbic system in the waking animal *Electroenceph clin Neurophysiol* 1953 *5* 91—100

NAUTA W J H Fibre degeneration following lesions of the amygdaloid complex in the monkey *J Anat (Lond)* 1961 *95* 515—531

RANSON S W H KABAT and H W MAGOUN Autonomic responses to electrical stimulation of hypothalamus preoptic region and septum *Arch Neurol Psychiat (Chicago)* 1936 *33* 467—477

SCHONBERG Om livmoderens bevægelsesnerver *Norsk Mag. f Lægevid* 1864 *18* 1034—1048

SHEALY C and J PEELE Studies on amygdaloid nucleus of cat *J Neurophysiol* 1957 *20* 125—139

SMITH W K Studies on the cingular and pyriform regions of the cerebral cortex *Trans Amer neurol Ass* 1949 *74* 169—171

TANG P C Levels of brain stem and diencephalon controlling micturition reflex *J Neurophysiol* 1955 *18* 583 595

URSIN H and B R KAADA Functional localization within the amygdaloid complex in the cat *Electroenceph clin Neurophysiol* 1960 *12* 1—20

UVNAS B Effect of frequency of hypothalamic stimulation upon bladder response *Proc Soc exp Biol (N Y)* 1947 *64* 181—185

WANG S C and F HARRISON The nature of bladder responses following stimulation of the anterior hypothalamus *Amer J Physiol* 1939 *125* 301—309

Acta physiol. scand. 1966. 66. 103—105

From the Department of Pharmacology, University of Goteborg, Sweden

The Effect of Triiodothyronine Treatment on the Catecholamine Content of the Blood during Infusion of Adrenaline in Man

By

Jan Häggendal and Nils Svedmyr

Received 29 April 1965

Abstract

Haggendal, J and N Svedmyr *The effect of triiodothyronine treatment on the catecholamine content of*

It has long been known that thyroid hormones enhance a number of adrenaline effects (reviews Ellis 1956, Hoch 1962, Harrison 1964, Rosenberg and Bastomsky 1965) Several explanations for this action have been presented One is that thyroid hormones influence the adrenaline inactivation in such a way that more amine is made available to the receptor mechanism Another explanation is that the sensitivity of the effector cells increases

In a previous study the first of these hypotheses was investigated in the rat (Svedmyr and Waldeck 1965), whereby no evidence was found to indicate that thyroxine affected the metabolism of injected catecholamine (CA)

Uptake in adrenergic neurons and breakdown, predominantly through catechol-O-methyl-transferase (COMT) appear to be the most important inactivating mechanisms for circulating CA (Axelrod *et al* 1959, Axelrod and Tomchick 1960, Whitby Axelrod and Weil Malherbe 1961 De Schaepdryver and Kirshner 1961, Carlsson and Waldeck 1963 Inhibition of either of these mechanisms leads to an increase in the quantity of circulating CA

Many attempts have been made to determine the effect of thyroid hormones on the CA content of the blood Older studies often showed a higher CA content in thyrotoxicosis or in animals treated with thyroid hormones (Harrison 1964) The reliability

References

BOCHEFONTAINE L T, Étude expérimentale de l'influence exercée par la faradisation de l'écorce grise du cerveau sur quelques fonctions de la vie organique *Arch Physiol norm path* 1876 *8* (Sér 2) 140—172

GJONE R, Peripheral autonomic influence on the motility of the urinary bladder in the cat I Rhythmic contractions *Acta physiol scand* 1965a *65* 370—377

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KOIKEGAMI H T DODO Y MOCHIDA and H TAKAHASHI Stimulation experiments on the amygdaloid nuclear complex and related structures effects upon renal volume urinary secretion movement of urinary bladder blood pressure and respiratory movements *Fol a psychiat neurol jap* 1957 *11* 157—206

KREMER W F Autonomic and somatic reactions induced by stimulation of the cingular gyrus in dogs *J Neurophysiol* 1947 *10* 371—379

KAADA, B R Somato-motor autonomic and electrocorticographic responses to electrical stimulation of rhinencephalic and other structures in primates cat and dog *Acta physiol scand* 1951 *24* (Suppl 83)

MAGNUS O and H J LAMMERS The amygdaloid nuclear complex *Folia psychiat neerl* 1956 *59* 555—582

MCLEAN P D and J M R DELGADO Electrical and chemical stimulation of frontotemporal portion of limbic system in the waking animal *Electroenceph clin Neurophysiol* 1953 *5* 91—100

NAUTA W J H Fibre degeneration following lesions of the amygdaloid complex in the monkey *J Anat (Lond)* 1961 *95* 515—531

RANSON S W H KABAT and H W MAGOUN Autonomic responses to electrical stimulation of hypothalamus preoptic region and septum *Arch Neurol Psychiat (Chicago)* 1936 *33* 467—477

SCHONBERG Om hvmoderens bevægelsesnerver *Norsk Mag f Lægevid* 1864 *18* 1034—1048

SHEALY C and J PEELE Studies on amygdaloid nucleus of cat *J Neurophysiol* 1957 *20* 125—139

SMITH W K Studies on the cingular and pyriform regions of the cerebral cortex *Trans Amer neurol Ass* 1949 *74* 169—171

TANG P C Levels of brain stem and diencephalon controlling micturition reflex *J Neurophysiol* 1955 *18* 583—595

URSIN H and B R KAADA Functional localization within the amygdaloid complex in the cat *Electroenceph clin Neurophysiol* 1960 *12* 1—20

UVNÄS B, Effect of frequency of hypothalamic stimulation upon bladder response *Proc Soc exp Biol (N Y)* 1947 *64* 181—185

WANG S C and F HARRISON The nature of bladder responses following stimulation of the anterior hypothalamus *Amer J Physiol* 1939 *125* 301—309

thyronine was chosen in consideration of the futher pulse rise that was to be expected

(Svedmyr 1965)

The A content of the plasma (Fig 1) before the A infusion was not affected by the triiodothyronine treatment, and in most of the subjects lay below the determination limit of the method rising during the A infusion to approximately 0 7 μg/litre (uncorrected for the recovery) The increase was of the same magnitude in the control and triiodothyronine experiments After discontinuation of the infusion, the A content decreased equally rapidly in both groups The NA contents were low, similar in the two groups and did not change noteworthily during the A infusion.

Discussion

The above results contradict the view that the potentiation of certain of the A effects obtained by pretreatment with triiodothyronine may be due to influence on the uptake of circulating CA or inhibition of those enzymes (predominantly COMT) which are of importance in the breakdown of circulating CA

This is in agreement with the results of Svedmyr and Waldeck (1965) obtained on the administration of tritium labelled CA in the rat It seems probable therefore that in those cases where potentiation of A effects was induced by triiodothyronine treatment this was brought about by a change on the part of the effector cell.

It should be pointed out, however that the dose of triiodothyronine used was relatively small and that the possibility of effects on the CA metabolism by higher thyroid hormone doses cannot be precluded.

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References

AXELROD F Metabolism of epinephrine and other sympatomimetic amines *Physiol Rev* 1959 *39* 751 776

AXELROD J and R. T TOMCHICK, Increased rate and metabolism of epinephrine and norepinephrine by sympatomimetic amines *J Pharmacol exp Ther* 1960 *130* 35 —369

CARLSSON A and B WALDECK, On the role of liver catechol-O-methyl transferase in the metabolism of circulating catecholamines *Acta pharmacol* (Kbh) 1963 *20* 4 55

DE SCHAEPDRYVER, A. F and N KIRSHNER The metabolism of DL-adrenaline-[2] [14]C in the cat. II — Tissue metabolism. *Arch. int Pharmacodyn* 1961 *131* 433—449

ELLIS, S The metabolic effects of epinephrine and related amines. *Pharmacol Rev* 1956, *8* 485—562

HAGGENDAL, J An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues *Acta physiol scand* 1963 *59* 242—254

HARRISON T S Adrenal medullary and thyroid relationships. *Physiol Rev* 1964 *44* 161 185

HOCH F L Biochemical actions of thyroid hormones *Physiol Rev* 1962 *42* 605—6 3

JONES, R. T and W D BLACKE Hyperthyroidism and the dynamics of distribution and renal excretion of epinephrine in the dog *Amer J Physiol* 1958 *193* 3 5—378.

ROSENBERG J N and C H BASTOMSKY The thyroid. *Ann Rev Physiol* 1965 2 1 105

SVEDMYR, N The influence of triiodothyronine treatment on some effects of adrenaline and noradrenaline in experiments on man. *Acta physiol scand* 1966 *66* In press

SVEDMYR N and B WALDECK. The effect of L-thyroxine treatment on the metabolism of tritium labelled catecholamines administered to the rat. *Acta pharmacol* Kbh. 1965 *23* In press

Acta physiol. scand. 1966. 66. 106—114

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Radiostrontium, Radiophosphorus, Stable Strontium, Stable Calcium, DNA and DNA-P in Ram Spermatozoa

By

Bertil Åberg and Margot Gillner

Received 4 May 1965

Abstract

Åberg B and M Gillner *Radiostrontium radiophosphorus stable strontium stable calcium DNA and DNA P in ram spermatozoa* Acta physiol scand 1966 *66* 106—114 — A detailed study was performed in order to determine the normal content of DNA DNA P Sr and Ca in ram spermatozoa Furthermore ^{89}Sr and ^{32}P were injected intravenously and their uptake by spermatozoa followed as well as their effect on the sperm cell picture The normal content of DNA in spermatozoa was found to be 2 9 pg per cell and the content of DNA P 0 21 pg per cell The ratio stable strontium to stable calcium in spermatozoa was 1 118 and the ratio stable strontium to DNA P 1 1 846 The ratio stable calcium to DNA P was found to be 1 18 After intravenous administration of radio strontium to rams a maximal concentration occurred in the spermatozoon on the 3rd to 7th day At that time the ratio ^{89}Sr DNA P was 0 25 10 [illegible] After i v administration of radiophosphorus to rams the maximal ratio of ^{32}P DNA-P was 0 17 10 [illegible] When 31 5 mCi of ^{89}Sr was i v administered to a ram the sperm cells were dead between the 45th and 52nd day Eight weeks after the administration the sperm cell picture was normalised

Luning *et al* (1963) reported genetic effects of strontium 90 injected into male mice i e increased number of intra uterine deaths within 5 weeks after the injections According to the literature nothing has been reported on radio-strontium uptake by spermatozoa and the relation between radiostrontium and the spermatozoal DNA

Material and methods

Rams (Swedish country breed were chosen because of their high sperm cell density in the semen (1 6 × 10[illegible]—3 7 × 10[illegible] cells per ml Three rams were used in these investigations and trained on an artificial vagina Ejaculates were collected from 1 to 3 times a week starting at least 4

Table I

Analytical procedure	$\bar{x} \pm \sigma$	Range	σ %
Sperm concentration	(2 6±0 2) 10[illegible] ml^{-1}	1 6 10[illegible]–3 7 10[illegible] ml^{-1}	7 5
Spermatocrit	24±3 %	12–41 %	12 3
DNA, mg per sperm cell	(2 9±0 3) 10[illegible]	2 0 10[illegible]–3 7 10[illegible]	9 7
DNA P, mg per sperm cell	(0 21±0 02) 10[illegible]	0 12 10[illegible]–0 36 10[illegible]	10 0
Deoxyribose mg per sperm cell	(0 56±0 09) 10[illegible]	0 31 10[illegible]–0 82 10[illegible]	16 1
Ca, atoms per sperm cell	(2 4±0 2) 10[illegible]	1 3 10[illegible]–3 1 10[illegible]	8 4

$\bar{x} = \frac{\Sigma x}{n}$ x = single observation d = difference between double determinations $\sigma = \pm \sqrt{\frac{\Sigma d^2}{2n}}$

n = number of observations $\sigma \% = \frac{\sigma \cdot 100}{\bar{x}}$

weeks before isotopes were administered Collections were continued during at least 8 weeks after the isotope administration

min in order to inhibit sperm motility

samples were centrifuged in a Beckman Microfuge and the plasma was removed with a teflon pipette (Sanz 1959) The sperm cells were transferred to ordinary glass tubes and washed 3

Portions of the sodium hydroxide solution of the isolated DNA were used for determination of DNA phosphorus according [illegible] and DNA determination by direct [illegible] nm respectively (Hutchison and Mu[illegible] [illegible]th a Hilger UVISPEK Trials mad[illegible] [illegible]eter showed less sensitivity

Standard solutions of DNA were prepared from thymus-DNA (Fluka Buchs Switzerland and a very pure DNA kindly given us by Professor Einar Hammarsten The Hammarsten

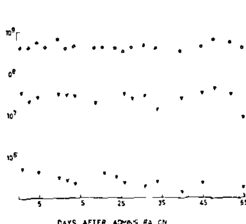


Fig 1

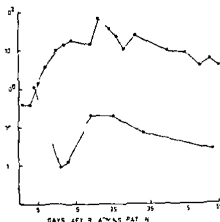


Fig 2

Fig 1 Atoms of DNA P Ca and Sr per spermatozoon
○ ram no 3 atoms DNA P per sperm cell
▽ ram no 3 atoms Ca per sperm cell
▼ ram no 3 atoms Sr per sperm cell

Fig 2 Atoms of P^{32} and Sr^{85} per spermatozoon
○——— atoms P^{32} per sperm cell ram no 3
●———● atoms Sr^{85} per sperm cell ram no 2

preparation being superior to others was used for final calculations of concentration 2 deoxy d ribose from Th Schuchardt München was used as deoxyribose standard As phosphate standard the analytical grade KH_2PO_4 from Merck was used

Radiostrontium and radiophosphorus were obtained from Amersham England ($Sr^{85}Cl_2$ SMS 1 $Sr^{85}Cl$ SOS 2 P^{32} as orthophosphate PBS 1) The isotopes were administered ntravenously The doses ... 10 0 mCi of

sional shakings 60 µl of the digest were diluted with 2 0 ml of an aqueous solution of $LaCl_3$ (1 per cent in 5 per cent HCl) The concentrations of calcium and strontium were determined with a Perkin Elmer model 303 Atomic Absorption spectrophotometer against standard solutions of $CaCl_2$ analytical grade) and $SrCl_2$ (analytical grade) containing $LaCl_3$ The detection limit with Scale 2 for calcium) was 0 05 mg/l and with Scale 10 (for strontium) 0 01 mg/l

The accuracy of the analytical procedures was calculated from double determinations and the results are given in Table 1

Results and discussion

The results of the strontium radiostrontium calcium, radiophosphorus DNA DNA P and deoxyribose determinations are given in fig 1—3 The values for elements stable and unstable, are given as number of atoms per spermatozoon so as to ensure an easy way of calculating relationships DNA DNA P and deoxyribose are expressed as mg per spermatozoon The number of DNA P atoms is also given The percentage of the dose of P^{32} administered per ml of semen spermatozoa and seminal plasma is

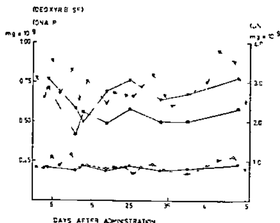


Fig 3 mg of DNA deoxyribose and DNA P per spermatozoon in two different rams

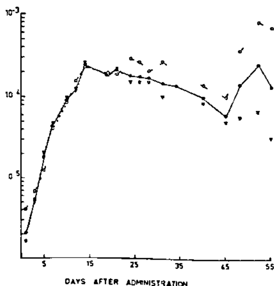


Fig 4 Per cent of given dose of P^{32} in the semen the spermatozoas and the seminal plasma in ram no 3

Ram no 3 — per cent of given dose P^{32} per ml spermatozoa
per cent of given dose P^{32} per ml semen
per cent of given dose P^{32} per ml seminal plasma

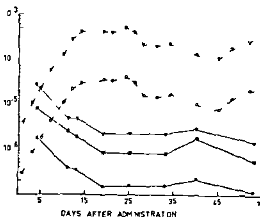


Fig 5

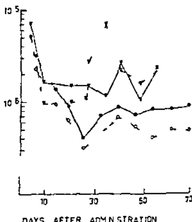


Fig 6

Fig 5 Per ce



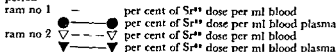


Fig 6 Blood and blood plasma concentrations of Sr^{89} in ram no 1 & 2 during the experimental period

ram no 1 — per cent of Sr^{89} dose per ml blood

●——● per cent of Sr^{89} dose per ml blood plasma

ram no 2 ▽– – –▽ per cent of Sr^{89} dose per ml blood

▼——▼ per cent of Sr^{89} dose per ml blood plasma

shown in Fig 4 The percentage of the dose of Sr^{89} per mg of DNA DNA P and deoxyribose is given in Fig 5 which also shows the relationship between Sr^{89} and P^{32}

The blood concentration of radiostrontium after the administration is shown as per cent of dose in Fig 6

Radiophosphorus was readily taken up by spermatozoa as already shown by Dawson (1958) (Fig 4) Radiostrontium was also taken up by the sperm cells A maximum in spermatozoal radiostrontium content in all 3 rams was reached between the 3rd and 7th day after the intravenous administration When Sr^{89} was administered the radio-isotope in spermatozoa was determined for 53 and 74 days respectively whereas after the administration of Sr^{89} the limit for determination was reached on the 13th day

Our values for spermatozoa DNA in rams (about 2 9 10 ⁹ mg/cell) are less than the value reported by Mann in 1954 (3 2 10 ⁹) The difference might be due to our use of NaOH as a solvent instead of KOH furthermore the standard DNA used in the determinations might differ in extinction coefficients Our values however do accord with those of *Walker and Yates (1952)*

The values for DNA P found by us (0 21 10 ⁹ mg/cell) are also less than Mann s in 1954 (0 36 10 ⁹)

Further investigations are necessary to establish if the decreased content of DNA & DNA P is a result of the radiostrontium administration In the present investigation the Sr/Ca ratios varied from 1/45 to 1/239 in sperm cells Our mean value was 1/118

TABLE II Ram I

Days after adm	Cytoplasmic droplets		Pathological head forms in per cent	Pathological midpieces in per cent	Looped tails in per cent	Acrosomal defects	Motility in per cent	Wave formation
	prox	dist						
−14			1.0				85	+++
0			0.6		2.6		80	+++
11			1.4	0.2	0.4		75	+++
18		1.5	2.2	0.2	1.4		65	+++
38	1.5	1.5	2.2	0.2	6.2		65	+++
45	1.0	1.0	11.0	1.5	21.5		0	0
52	11.5	13.5	22.2	0.4	12.0	—	<5	0
59			7.4	0.4	0.6		30	+[1]
68			1.8	0.2	0.4		75	+++
74			0.8	0.4	0.2		80	+++

[1] Decapitated sperm cells 2.6 %

The ratios for Sr/DNA P and Ca/DNA P were respectively 1/1846 and 1/18 (Fig 1). Sr^{89}/DNA P was found to be $1/4 \cdot 10^{8}$ and Sr^{85}/DNA P $1/4 \cdot 10^{8}$ at the maximum level in spermatozoa (3—7 days after administration of the isotope). The ratio P^{32}/DNA P was $1/6 \cdot 10^{8}$ at the maximum P^{32} level. These quotients are of some interest because it has been shown that yttrium has a considerable affinity for DNA (Stern and Steinberg, 1953) and this affinity of yttrium also pertains to proper lanthanides (cf Magnusson 1963). The unstable strontium isotopes Sr^{89} and Sr^{90} decay to yttrium whereas Sr^{85} decays to Rb^{85} but we are interested only in Sr^{89} and Sr^{90} as fission products of physiological importance.

As is evident from Fig 2 both Sr^{89} and P^{32} in spermatozoa show a peak on about the 20th day which must be due to an uptake which has occurred in the testicles. Dawson (1958) has shown that the passage time from the testicles through the epididymis to the ejaculate is roughly that time.

The analytical values here presented clearly show that strontium is present in spermatozoa and that radiostrontium circulating in the ram's blood stream is taken up by spermatozoa. The decay of unstable strontium atoms and their daughters will naturally affect the DNA in the cell. This must be the case particularly for yttrium which is attached to the DNA presumably at the phosphorus atoms. Thus radiostrontium taken in by the animal organism from fall out will add to the dose received by the spermatozoal radiocesium (Cs^{137}) (Ekman and Greitz 1965).

That radiostrontium affects the spermatozoa is obvious from Table II which shows the percentage of live sperm cells in ejaculates from the ram which received 31.5 mCi of Sr^{89}. In the 6th and 7th weeks after the administration no wave formation was found

TABLE III Ram I

Days after adm	Hematocrit % cells	Hb g/100 ml	White blood cells/mm³	Neutrophilic Band Rel %	Neutrophilic Band No of cells
0	33	11 0	5 900		
7	28	9 7	3 100	1 5	47
15	27	8 4	3 000		
33	25	8 1	4 300	0 5	22
	Some basophilic red blood cells				
40	27	9 3	3 000		
	Anisocytosis				
47	31	9 3	4 200		
	Anisocytosis some Howell Jolly bodies				
54	31	10 9	3 500		
	Slight anisocytosis				
62	34	10 8	4 000		
	1 nucleated red blood cell Howell Jolly bodies				
70	33	11 4	2 600		

TABLE IV Ram II

Days after adm	Hematocrit % cells	Hb g/100 ml	White blood cells mm³	Neutrophilic Band Rel %	Neutrophilic Band No of cells	Neutrophilic Segmented Rel %	Neutrophilic Segmented No of cells
5	32	10 1	8 200			23	1 886
12	29	10 3	9 800	0 5	49	54 5	5 341
20	31	10 5	6 300			26 5	1 670
26	30	10 1	6 700	0 5	34	9	603
34	31	10 8	5 200			11	572
40	24	9 7	6 500			8 5	553
48	30	10 5	4 200			7 5	315
55	30	10 8	3 500	0 5	18	4	14

in the ejaculates and the dead cells showed a great number of pathological heads In the 8th week the motility was still greatly reduced Hereafter motility returned and was normal from the 9th week when the number of pathological types of sperm cells was also normal

Segmented		Eosinophils		Basophils		Lymphocytes		Monocytes	
Rel %	No of cells	Rel %	No of cells	Rel %	No of cells	Rel %	No of cells	Rel %	No of cells
10	590	1	59			88 5	5 722	0 5	30
26	806	3	93			66 5	2 062	3	93
30	900	1	30			69	2 070		
13	559	1	43			85 5	3 677		
12 5	375					87	2 610	0 5	15
19	798					81	3 402		
1 5	543	0 5	18			84	2,940		
7	280	2	80			90 5	3 620		
5	130	2 5	65			92 5	2,405		

Eosinophils		Basophils		Lymphocytes		Monocytes		Juveniles	
Rel %	No of cells	Rel %	No of cells	Rel %	No of cells	Rel %	No of cells	Rel %	No of cells
11	902			66	5 412				
8	784			36 5	3 577			0 5	49
2 5	158			71	4 473				
1	67			88 5	5 930			1	67
1	52			87	4 594	1	52		
2 5	163			89	5 785				
				91 5	3 843	1	49		
0 5	18			94 5	3 308	0 5	18		

Radiostrontium in the dose here applied 31 5 mCi of Sr^{89} in ram I and 10 mCi of Sr^{89} in ram II) also caused clinical changes Thus both animals developed leukopenia and neutropenia Table III and IV and ram II died on the 61st day On post mortem examination this ram showed definite signs of a radiation syndrome The first ram was

TABLE III Ram I

Days after adm	Hematocrit % cells	Hb g/100 ml	White blood cells/mm³	Neutrophilic Band Rel %	Neutrophilic Band No of cells
0	33	11 0	5 900		
7	28	9 7	3 100	1 5	47
15	27	8 4	3 000		
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TABLE IV Ram II

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5	32	10 1	8 200			23	1 886
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20	31	10 5	6 300			26 5	1 670
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48	30	10 5	4 200			7 5	315
55	30	10 8	3 500	0 5	18	4	14

in the ejaculates and the dead cells showed a great number of pathological heads In the 8th week the motility was still greatly reduced Hereafter motility returned and was normal from the 9th week when the number of pathological types of sperm cells was also normal

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From the Department of Physiology and Women's Clinic University of Turku, Finland

Sulphate Conjugation during Development, in Human, Rat and Guinea Pig

By

MARTTI O PULKKINEN

Received 10 May 1965

Abstract

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tion and analysis of the conjugates was carried out by determining the sulphate form as a complex with methylene blue in chloroform

The sulphurylation appeared to be carried on faster as the animal developed and this phenomena does not appear to be dependant on the animal species or on the tissue used in the experiments The sulphate conjugation of phenols may develop more rapidly, in the rat after the age of two weeks than that of steroids

The role of sulphate conjugation as a detoxifying factor for phenols and as a changer of steroid structure has been known for a long time The main ideas of the present view of sulphate conjugation were introduced by Lipmann (1958) When studying the oestrogen metabolism of the foetoplacental unit, it has been possible to show the importance of this type of conjugation both for the foetus and the mother This could show in some foetal tissues that oestradiol, oestrone and oestriol can conjugate with sulphate (Diczfalusy *et al* 1961) It was also interesting to note how this kind of function changed in the period from intrauterine life to maturity, also e g with bilirubin and drugs

The other important form of conjugation to note is the β-d glucosiduronic acid conjugation which has already been quite widely studied in this respect (Hartiala and Pulkkinen 1964) From a clinical viewpoint there is in some cases a low concentration of uridinediphosphoglucuronic acid transferase in newborn infants and it has been shown that this is important in the aetiology of icterus Glucuronide conjugation in the foetus was considered in 1955 by Hartiala and Pulkkinen

TABLE I The development of sulphate conjugation in the tissues of rat and guinea pig Results number of determinations, carried out

	Liver	
	Rat	Guinea pig
p nitrophenol under 4 g	2 7±0 9 (9)	—
over 4 g	4 8±1 3 (8)	—
new born	13 4 (3)	—
1—4 days	10 1±1 4 (9)	24 9±8 3 (9)
14 days	23 3 (1)	75 3 (2)
30 days	39 2±4 0 (8)	93 3 (2)
90 days	45 1±5 8 (10)	57 1±4 6 (14)
dehydroepiandrosterone foetus	0 8±0 7 (6)	1 9±0 7 (15)
new born	4 4 (2)	—
1—4 days	14 6±1 7 (9)	14 0±5 0 (9)
14 days	15 1 (1)	55 3 (2)
30 days	29 3±3 5 (8)	38 8 (2)
90 days	27 8±2 8 (10)	40 9±3 8 (14)
oestrone foetus	0 3±0 2 (6)	0 6±0 3 (12)
new born	—	5 0±2 4 (8)
14 days	2 5 (1)	21 7 (2)
30 days	4 4±0 8 (8)	12 6 (2)
90 days	7 3±1 0 (10)	13 2±1 8 (14)

Owing to the fact that we are concerned here with an important physiological function the conjugation of p-nitrophenol, dehydroepiandrosterone and oesterone with sulphate in the developing tissues of human, rat and guinea pig were studied Preliminary results were published in a Congress Abstract 1963

Materials and methods

The test material consisted of 42 guinea pigs 48 Wistar rats and 5 human foetus see Table I The size of the material from the human (foetal development) was limited to 19 cm Among the different organs studied were liver lung kidney intestine, suprarenals and brain, but of ... was

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ultramix" homogeniser for 15 sec and this was further homogenised in a Potter Elvehjelm homogeniser at 4 C One part of the first homogenate was centrifuged with 3 parts EDTA ...

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— so a more marked conjugation can be expected as foetal tissues are being used Howe r the conjugation capacity is small and the weight of tissue used is small), this is why the modifica-

expressed as μg conjugated phenol or steroid per g wet weight per hour In parenthesis, the

Kidney		Lungs	
Rat	Guinea pig	Rat	Guinea pig
0 4 (2)	—	—	—
0 9	—	1 8±0 8 (7)	—
2 1 (1)	—	6 2 (3)	—
1 1±0 3 (8)	4 3±1 0 (3)	2 4±0 6 (9)	10 7±3 3 (7)
8 9 (1)	8 6 (2)	1 2 (1)	17 9 (2)
5 9±1 3 (8)	3 4 (1)	2 6±0 8 (4)	5 9 (2)
6 8±2 7 (10)	5 8±1 4 (8)	4 1±1 3 (6)	6 4±1 1 (7)
—	3 0±1 1 (11)	1 1±0 7 (5)	0 7±0 3 (12)
—	—	—	—
1 9±0 4 (6)	2 9 (2)	3 8±0 8 (9)	4 5±2 4 (7)
8 1 (1)	7 1 (2)	5 9 (1)	4 4 (2)
3 3±1 5 (8)	5 9 (1)	4 6±1 9 (4)	5 5 (2)
0 9±0 4 (10)	4 8±1 5 (8)	3 6±0 6 (6)	3 3±1 0 (7)
—	1 2±0 6 (6)	1 9 (1)	0 9±0 7 (11)
—	—	—	4 3 (1)
—	—	—	2 3 (1)
—	—	—	1 6 (2)
0 6 (2)	1 3±0 6 (8)	—	0 9±0 6 (7)

tion was necessary With the precipitate obtained here the conjugating activity is stable for at least one day if this is stored at — 15° C a technique not previously used The precipitative was dissolved in water in a concentration relative to 1 g of original tissue per ml phosphate buffer

The enzyme 0 4 ml and 0 1 ml substrate were incubated for 2 hrs at 37° C with 0 5 ml

Results

The results were calculated as μg conjugated phenol or steroid per gram wet weight/ hour Results for the development of conjugation in rat liver are shown in Fig 1, and Table I and II show the results with other tissues and animal strains It was observed that as the age increases the capacity for sulphate conjugation with phenols and steroids also increases This was observed to be the same with all three strains used The sulphate conjugation of phenols may develop more rapidly in the rat after the age of two weeks, than that of steroids

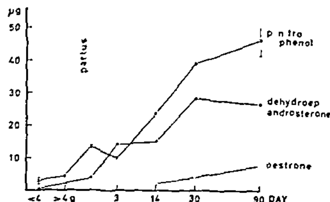


Fig 1 The development of sulphate conjugation in rat liver The results as μg conjugated phenol or steroid per gram wet weight/hour

TABLE II The development of sulphate conjugation in human foetal tissues Results expressed as μg of conjugated phenol or steroid per g wet weight per hour In parenthesis the number of determinations carried out

	p-nitrophenol	Oestrone	Dehydro-epiandro-sterone
Lenght of foetus less than 10 cm			
liver	6 8 (1)		
lungs	4 9 (1)		
intestine	4 5 (1)		
kidney	6 4 (3)		
Lenght of foetus more than 10 cm			
liver	15 4 ± 2 3 (7)	21 6 ± 5 8 (5)	9 3 (3)
kidney	10 6 ± 2 5 (6)		–
suprarenal glands	9 3 (3)		
lungs	8 0 ± 1 4 (7)	5 7 ± 0 4 (4)	6 9 (2)
intestine	8 9 ± 1 6 (7)	12 1 ± 4 9 (3)	11 1 (1)
brain	1 5 (3)	2 7 (3)	1 4 (3)

Discussion

The results of these observations of sulphate conjugation are not analogous to those seen with glucuronide conjugation It can be noted that at the age of one month the activity is no stronger than in the adult This could be seen definitely to be the case with glucuronylation (Hartiala and Pulkkinen 1964) There are large differences in the speeds of conjugation between different organs and tissues Quantitatively p-nitrophenol and dehydroepiandrosterone can be conjugated more readily than oesterone in the rat

The mechanism of sulphate conjugation for steroids can be settled only when it has been made clear what the differences are between the biological activities of the free and conjugated forms The biological effect is probably different, but despite this there is still no knowledge available In addition to this we do not know how the monosulphates can differ in their effect from e g disulphates Although this kind of sulphurylation mechanism is thought to be well developed during foetal life, it is seen that in comparison with adults there are many "defects" in the foetus In the light of this work it can be seen that the foetus has not a very high capacity to form sulphate conjugates This is analogous to the formation of the glucosiduronic acid conjugate during the foetal period (Hartiala and Pulkkinen 1955)

If the results obtained here are compared with those of Wengle (1964) the same observation can be made, i e that as age increases so does conjugation capacity The method used in this work needs much tissue despite the modification of the ammonium sulphate fractionation, so it has not been possible here to analyse the steroid sulphokinase activity in suprarenal glands It has only been possible to study with all the tissues the conjugation of phenol, which shows up more clearly in this method There was not enough tissue to enable us to study many substrates We perhaps cannot consider the conjugation capacity of rat foetus to be absent although it is lower than in the newborn infant The results show that the speed of conjugation was almost the same in both works The tendency in this work to a little lower capacity could be due to e g the length of incubation time and other differences in reaction conditions

The conjugation of p nitrophenol seems to go up after the first two weeks of life relatively more than that of the steroids This was observed here and also by Wengle using only male animals In regard to age development there seem to be large differences between phenolsulphokinase and steroidsulphokinase activity

References

DICZFALUSY E O CASSMER C OLONSO and M DE MIGUEL Oestrogen metabolism in the human foetus I Tissue levels of following the administration of 17 β-oestradiol and oestriol *Acta endocr* (Kbh) 1961 *37* 353—375

HARTIALA K J W and M PULKKINEN Studies on detoxication mechanism IV Glucuronide synthesis in foetal rabbits *Ann Med exp Fenn* 1955 *33* 246—248

HARTIALA K J W and M O PULKKINEN Studies of the possible role of sex hormones on the change in conjugation capacity at various ages in rat I Comparison of total glucuronide conjugation UDPGA transferase and β glucuronidase activity in growing rats. *Ann Acad Sci fenn* 1964 106/12

LIPMANN F Biological sulfate activation and transfer Studies on a mechanism of group activation and its role in biosynthesis are described *Science* 1958 *128* 575—580

NOSE Y and F LIPMANN Separation of steroid sulphokinases. *J biol Chem* 1959 *233* 1348—1351

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Acta physiol. scand. 1966, 66, 120—122

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Sulphate Conjugation during Pregnancy and under the Influence of Cortisone

By

MARTTI O. PULKKINEN

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Abstract

PULKKINEN, M O *Sulphate conjugation during pregnancy and under the influence of cortison.* Acta physiol scand 1966 *66* 120—122 — During pregnancy there is a tendency to lower sulphate conjugation with p-nitrophenol and also perhaps a tendency to lower sulphurylation of the steroids in guinea pig and rat Cortisone has no influence on the sulphate conjugation capacity of these compounds in non pregnant rats The sulphurylation capacity of placenta is very low also and the balance between conjugation and hydrolysis can be assumed to be on the side of the latter

From certain endocrine factors, it has been observed that pregnancy has an influence on glucuronylation (Rauramo *et al* 1963), and also steroids are able to inhibit conjugation both in vitro and in vivo Cortisone can inhibit the conjugation of glucosiduronic acid and o-aminophenol in the gastrointestinal tract (Halme *et al* 1959) The ease with which p-nitrophenol, dehydroepiandrosterone and oestrone can conjugate with sulphuric acid has been studied in the pregnant guinea pig and rat, and also in the placenta The experiment was carried out in order to clarify how cortisone can influence there phenomena because the concentration of cortisone is very high during pregnancy

Materials and methods

[illegible] 10 placentas [illegible] ect [illegible] rat [illegible] ilk [illegible]

Results

The results during pregnancy are listed in Table I and II It can be considered that the rat and guinea pig in both liver and kidney, have a lowered sulphate conjugation

TABLE I The sulphate conjugation during pregnancy in rat and guinea pig Results expressed µg conjugated phenol or steroid per g wet weight per hour In parenthesis the number of determinations carried out

Phenol or steroid	p-nitrophenol	Oestrone	Dehydroepiandrosterone
Rats, pregnant			
liver	28 1±2 8 (16)	6 2±0 7 (14)	32 3±3 3 (14)
lungs	3 7±1 6 (7)	2 3±1 4 (5)	2 0±1 1 (7)
kidney	3 8±1 7 (8)	—	0 9±0 4 (8)
Rats, non pregnant			
liver	45 1±5 8 (10)	7 3±1 0 (10)	27 8±2 8 (10)
lungs	4 1±1 3 (6)	—	3 6±0 6 (6)
kidney	6 8±2 7 (10)	0 6 (2)	0 9±0 4 (10)
Mothers of new born rats			
liver	43 5±17 6 (7)	8 6±5 4 (5)	21 1±2 7 (5)
lungs	4 1±2 7 (3)	—	1 8±1 5 (3)
kidney	4 7±2 5 (3)	—	0 9±0 5 (3)
Guinea pigs, pregnant			
liver	39 5±14 1 (16)	10 7±5 5 (13)	27 8±2 7 (13)
lungs	7 8±2 7 (13)	2 9±1 3 (11)	6 4±2 9 (13)
kidney	2 5±0 8 (11)	1 4±0 4 (10)	1 6±0 9 (11)
Guinea pigs, non pregnant			
liver	57 1±4 6 (14)	13 2±1 8 (14)	40 9±3 8 (14)
lungs	6 4±1 1 (7)	0 9±0 6 (7)	3 3±1 0 (7)
kidney	5 8±1 4 (8)	1 3±0 6 (8)	4 8±1 5 (8)

capacity, both for p-nitrophenol and for dehydroepiandrosterone No statistical differences were found with the low rate of conjugation of oestrone The conjugation in the placentas appeared to be negative or under the lowest limits of the method (p-nitrophenol 2 3 ± 1 1, oestrone 1 1 ± 1 0 and DHA 1 8 ± 0 9 µg/g wet weight per hour, in guinea pig) With cortisone no statistically significant effect on the sulphate conjugation could be seen

Discussion

During pregnancy the amount of corticosteroids in the plasma rises, and the excretion of these is much greater than normal However these corticosteroids that increase are the free corticosteroids and not the conjugated form (Pekkarinen *et al* 1962) There could be an explanation for this phenomenon if the conjugation changes or the hydrolysis of already conjugated compounds is increased, or both The author has studied the hydrolysis earlier (Pulkkinen and Paunio 1963) and found that there are not very marked changes in the maternal tissues but the hydrolysing capacity of the placenta

Reserpinization was effected in three animals by the i p injection of 7 mg Serpasil (Ciba) per kg b w 24 hrs before killing All the tests designed to establish the specificity of the fluorescence were used (cf Falck and Owman 1965) The various tissues were identified by phase-

Results

Judged from the histochemical criteria (cf Falck and Owman 1965) all the structures described here as adrenergic contained a primary catecholamine capable of condensing with formaldehyde into a fluorescent product, emitting a green to yellow-green light in the fluorescence microscope Tissues treated in the same way but without formaldehyde gas did not show any such specific fluorescence

In the *cornea*, there is a sparse occurrence of varicose adrenergic fibres (Fig 1) The amount varies from animal to animal, but none has been found to be without The adrenergic fibres mainly occur in the outer half of the cornea, although they do not form any definite layer Both isolated, fine, varicose fibres can be seen, as well as large trunks of myelinated nerve fibres containing several beaded adrenergic fibres These trunks run mainly radially, being thickest near the limbus, and divide dichotomously towards the vertex The adrenergic nerves are only very seldom seen in contact with blood vessels There are no intraepithelial fibres, nor are there any in or at the endothelium

In the *limbus*, there is the ordinary plexus of varicose adrenergic fibres around the vessels, that is, the nerves are superimposed upon the media as is the general pattern elsewhere in the body There is also a thin plexus of varicose adrenergic fibres in the connective tissue between the vessels, partly lying in trunks mainly directed towards the cornea, partly lying as a delicate lattice of fine fibres in the stroma

In the *sclera*, adrenergic fibres have been found only where vessels or nerves penetrate The autofluorescence is, however, somewhat disturbing, and may disguise some fine adrenergic fibres At the aqueous drainage paths the adrenergic fibres are very sparse A ciliary artery surrounded by the ordinary adrenergic plexus can occasionally be seen to pass through the sclera in this region It is at times accompanied by a loop from a nerve trunk (without any specific fluorescence) originating in the ciliary plexus A small nerve trunk containing no fluorescent fibres is often seen running equatorially in the middle of the sclera at the limbus, accompanied by a small vessel

In the loose trabecular meshwork of the *iridocorneal angle*, practically every tissue strand seems to contain at least one varicose adrenergic fibre The density of the plexus seems to be the least in the anterior uveal part (towards the anterior chamber), somewhat higher in the scleral meshwork and increasing still more farther backwards into the filtration cleft The amount of varicose adrenergic fibres seems to be generally somewhat higher in those parts lying close to the sclera The fibres run in all directions, although mainly following the strands of connective tissue in the frontal parts and the strands of meridional smooth muscle in the rear parts (Fig 2) Scattered in the loose connective tissue of the filtration cleft there is a number of rounded, intensely autofluorescent melanophores Two types can be distinguished, one with a bright, yellow autofluorescence and one with a somewhat more golden orange colour Both types are



Fig 1 Cornea, outer half, cat At the top of the picture the epithelium, and beneath some adrenergic nerve fibres × 215

Fig 2 Ciliary body cat At the top the ciliary epithelium with a subepithelial plexus of adrenergic nerve fibres At A the frontal part of the ciliary muscle Arrows point to some meridionally running tissue strands with varicose adrenergic fibres in the "filtration cleft" At the bottom, the somewhat autofluorescent sclera is seen There are no melanphores in this section × 175



Fig 3 Ciliary body ciliary processes, and iris, cat. The bright orange structures are autofluorescent melanophores Under the ciliary epithelium there is a bright greenish layer of adrenergic nerve fibres Below this layer lies the ciliary muscle, almost devoid of adrenergic nerve fibres At the bottom of the picture is seen the innervation of the tissue strands in the somewhat collapsed "filtration cleft' (cf Fig 2) The sclera has detached × 100



Fig 4 Iris papillary part cat The iridic stroma is filled with brightly autofluorescent orange melanophores Two layers of varicose adrenergic fibres are seen the frontal broader one represents the sphincter and the posterior one represents the dilatator Connections between the two layers are only few × 100



Fig 5 Nictitating muscle, cat. The adrenergic nerve fibres form a rather wide meshed plexus × 230

Fig 6 Lacrimal gland cat The acini are surrounded by a loose meshwork of adrenergic varicose nerve fibres × 135

finely granular in their cytoplasm, and neither contain any monoamines, as no decrease of the fluorescence intensity occur upon reserpinization or borohydride reduction In addition, ordinary, branched, dark, non fluorescent melanophores can be seen At times, a nerve trunk containing an occasional varicose adrenergic fibre can be seen running from the suprachoroid to the iris

In the *ciliary body*, only few fibres have been found in the main smooth muscles except in the posterior part, where some meridionally directed varicose adrenergic nerve fibres can be seen, without any apparent connection to anything but the muscle cells In the connective tissue strands and along the muscle bundles extending into the filtration cleft", varicose adrenergic fibres occur as described above (Fig 2) Near the sclera, adrenergic fibres in such muscle bundles can be seen to extend as far back as to the ora serrata Under the ciliary epithelium, there is a thick network of varicose adrenergic fibres surrounding the vessels in the ordinary way, and also located between these and the epithelium, but never within the latter Principially the same arrangement is seen in the slender ciliary processes, the density being less than in the ciliary body, however (Fig 3)

In the *iris*, the majority of the varicose adrenergic fibres lie within the muscular layers but also to some extent immediately frontal to the dilator A detailed study of the fluorescent fibres of the iris is in many parts prevented by the strong accumulation of [illegible]

fibres seem mostly to run isolated from each other The innervation pattern is the same through the whole muscle, also in parts extending to the iris root The varicose adrenergic fibres of the dilator muscle can be seen to lie as a discrete layer behind the sphincter, extending almost to the pupillary border, with only few and minute adrenergic varicose nerve connections between the two muscles The arteries of the iris have the same type of adrenergic nerve sheath as has been described in rabbits (Ehinger 1964 b), that is the adrenergic varicose fibres lie in a loose and spacious layer in the inner, loose part of the connective tissue sheath of the vessels In spite of the mentioned autofluorescence of the melanophores some few varicose adrenergic fibres can be found running in between these cells Sometimes, they come in close contact with the melanophores, but are more numerous towards the frontal border in contrast to the distribution of the melanophores which occur evenly distributed throughout the iridic stroma

In the *choroid* the tapetum has a very intense yellow autofluorescence No fibres have been found between this layer and the retina and in the choroid as a whole, the supply of varicose adrenergic fibres is sparse and mainly found in connection with vessels

In the *retina* there is a layer of varicose adrenergic fibres situated between the inner nuclear and inner plexiform layers Some of these fibres issue to form baskets of synaptic structures around non fluorescent ganglion cells in the inner nuclear layer Adrenergic cell bodies can also be seen in the inner part of the inner nuclear layer, but in amounts less than what is the case in e g cynomolgus monkeys rats rabbits or guinea pigs Further fluorescent perikarya can be seen in the ganglion cell layer often giving off a cone shaped process towards the layer of adrenergic fibres of the inner nuclear layer These cells occur with a frequency of about one in ten to one in twenty adrenergic

perikarya of the inner nuclear layer They are of moderate size and do not belong to the class of giant ganglion cells

In the *ciliary ganglion* (serial sections) or in the *optic nerve*, no varicose adrenergic fibres or adrenergic cells have been detected except around the vessels where the usual sheath is found Occasionally varicose adrenergic fibres can be seen in the nerve trunks connected to the ciliary ganglion

In the *eyelid* there is a plexus of varicose adrenergic fibres throughout the tarsal muscle The meshes are wide, and seem to engulf several muscle cells and thus it cannot be stated with certainty that every muscle cell receives an adrenergic fibre In the muscle of the nictitating membrane, there is a similar, relatively dense plexus of adrenergic nerve fibres (Fig 5)

In the arrectores pilorum, a rather dense network of varicose adrenergic fibres occur throughout the muscle Every muscle cell seems to be supplied with at least one nerve fibre No adrenergic fibres have been found in association with the tarsal glands (of Meiborn) or the sudoriferous glands (of Moll)

The *lacrimal glands* contain a network of varicose adrenergic fibres among the acini forming an autonomous ground plexus in the sense of Hillarp (1959) (Fig 6) The fibres lie close to the base of the acinar cells, but are never seen in or between them The density is approximately equivalent to that seen in the submaxillary gland of the rat and in the lacrimal and some salivary glands in the cynomolgus monkey (Ehinger 1965 a) The same type of innervation is seen in all parts of the lacrimal glands also in those of the nictitating membrane

Outside the retina no monoaminergic cell bodies have been identified After the reserpinization or after cervical sympathectomy, all the adrenergic fibres disappear except in the retina in the case of sympathectomy

Discussion

The finding of adrenergic varicose nerves in the cat cornea is in accordance with results from other species primates possibly excluded (cf Ehinger 1964 a and b 1965 a and Laties and Jacobowitz 1964) Phase contrast microscopy as well as vessel injection experiments (Ehinger 1965 b) have failed to demonstrate any significant relation between pathologically ingrown vessels and the adrenergic nerves The adrenergic fibres also occur regularly (in larger amounts than in the adult animal) at and before birth (Ehinger 1965 b) which excludes their presence being the result of any pathological changes The majority of the adrenergic fibres seem to be located within nerve trunks It is not clear whether they have any biological significance at all whether they should be interpreted as some kind of *nervi nervorum*, or whether they are intended for some stromal element of the cornea In embryonic guinea pigs and in new born dogs varicose adrenergic fibres have been found to lie intraepithelially in the cornea in addition to a rich plexus in the stroma (Ehinger 1965 b), a finding which does not favour a presumption that all adrenergic fibres of the embryonic cornea should be *nervi nervorum*

The supply of varicose adrenergic fibres to the trabeculae of the "filtration cleft with an increase in amount towards the sclera indicates that adrenergic nerves may influence the regulation of the aqueous flow The varicose adrenergic fibres in the posterior parts of the filtration cleft may have their influence on smooth muscle cells accompanying the trabecular strands but this cannot be the case further forward

where only connective tissue and endothelial cells occur The adrenergic varicose fibres are more abundant in the iridocorneal angle in the cat than in the rabbit but they do not equal the amount found in guinea pigs (Ehinger 1964 b) However, the interpretation of the functional meaning of the anatomical differences will have to await unambiguos results on the pharmacology and physiology of the aqueous outflow in the cat and in the guinea pig

The nerve supply to the dilatator pupillae is heavily adrenergic, as could be expected from classical physiology It is noteworthy that the amount of adrenergic fibres is larger than in other species studied (rats mice guinea pigs rabbits dogs cynomolgus and cercopithecus ethiops monkeys and humans, Ehinger 1964 a and b and 1965 a and b) The varicose adrenergic fibres can be found among the muscle cells as in rabbits and cynomolgus monkeys, in contrast to what has been found in rats and guinea pigs where they lie in front of the muscle In the cat sphincter, the number of fluorescent fibres is far more than expected and exceeds considerably the amount found in other species where only a moderate to sparse supply of varicose adrenergic fibres have been noted (Ehinger 1964 a and b and 1965 a, Laties and Jacobowitz 1964) The observation readily explains why adrenergic receptors have been demonstrated convincingly in the cat sphincter (Schaeppi and Koella 1964 Takats 1964) whereas the results from investigations in many other species have been more inconstant and indefinite β adrenergic receptors were claimed to occur in the cat sphincter while those of the dilatator should be α adrenergic The observed morphological separation of the adrenergic fibres in the dilator from those in the sphincter is of special interest in this connect on since it may indicate that a single adrenergic terminal nerve twig generally does not reach the receptors of both the dilator and the sphincter pupillae

The stromal varicose adrenergic fibres of the iris of primates have tentatively been claimed to innervate the spider shaped weakly reddishly autofluorescent melanophores (Ehinger 1965 a) In the cat the melanophores are of a dissimilar type in the respect that they are rounded, almost without processes and possessing an intense yellowish or orange yellow autofluorescence Their distribution is fairly even throughout the iridic stroma, whereas the adrenergic fibres are few and tend to accumulate towards the front of the iris Although adrenergic varicose fibres occasionally can be seen to come in close contact with autofluorescent melanophores it seems less likely that the brightly autofluorescent melanophores of the cat are adrenergically innervated

The adrenergic nerve pattern of the iris vessels is of a kind not usually found else where in the body, i e with a spacious loose adrenergic nerve sheath instead of the compact one commonly found superimposed upon the media As has been discussed previously (Ehinger 1964 b 1965 a) this is presumably due to the particular mesen chymal vessel sheath in the iris

In the ciliary body the distribution of adrenergic nerves to the smooth muscles differs from that of rabbits guinea pigs cynomolgus monkeys and humans which all have adrenergic nerves to all parts of the muscle (Ehinger 1964 b 1965 a) In the cat adrenergic fibres are found almost solely in the meridional muscle cells In dogs there is an arrangement resembling that of the cat Ehinger 1965 b

In the retina adrenergic fibres and nerve cells are found in a thin layer between the inner nuclear and the inner plexiform layers as has been described in rabbits rats and guinea pigs (Malmfors 1963 Haggendal and Malmfors 1963 The fluorescent perikarya of the adrenergic layer are rather sparse as compared with e g rabbits guinea pigs or rats Furthermore in the ganglion cell layer there occur some adrenergic ganglion

cells as described in rabbits, guinea pigs, and cynomolgus monkeys (Ehinger 1965 a) They are connected to the layer of adrenergic fibres by a rather coarse process Under certain conditions a special layer of adrenergic fibres has been demonstrated in the middle of the plexiform layer of rabbits, guinea pigs, and rats, and a third layer between the inner plexiform and ganglion cell layers in rabbits (Ehinger 1965 a and b), but there are no corresponding layers in normal cats

In the lacrimal glands the striking differences between the different species should be noted in cynomolgus monkeys (Ehinger 1965 a) and cats an adrenergic supply has been demonstrated whereas no such fibres have been found in rabbits rats or guinea pigs (Ehinger 1964 b)

The muscle of the nictitating membrane is generally regarded as being a special part of the tarsal muscle (cf Prince *et al*, 1960) There is also a similarity in distribution of varicose adrenergic fibres to the two muscles As has been noted in other species (Ehinger 1964 b 1965 a) the innervation is of a wide meshed type in comparison to that seen e g in the dilatator pupillae or in the vas deferens (Falck 1962, Owman and Sjostrand 1965) The meshes thus seem to engulf several muscle cells and it is not immediately evident that every individual muscle cell receives an adrenergic fibre.

References

EHINGER B Distribution of adrenergic nerves to orbital structures *Acta physiol scand* 1964 a *6?* 291—292

EHINGER B Adrenergic nerves to the eye and its adnexa in rabbit and guinea pig *Acta Univ Lund* Sectio II 1964 b No *20* 1—23

EHINGER B Adrenergic nerves to the eye and to related structures in man and in the cynomolgus monkey (*Macaca irus*) *Invest Ophthal* 1965 a In press

EHINGER B To be publ 1965 b

FALCK B Observations on the possibilities of the cellular localization of monoamines by a fluorescence method *Acta physiol scand* 1962 *56* Suppl 197

FALCK, B and CH OWMAN A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines *Acta Univ Lund* Sectio II 1965 No 7 1—23

HÄGGENDAL J and T MALMFORS Evidence of dopamine-containing neurons in the retina of the rabbit *Acta physiol scand* 1963 *59* 295—296

HILLARP N Å The construction and functional organization of the autonomic innervation apparatus *Acta physiol scand* 1959 *46* Suppl 157

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SCHAEPPI U and W P KOELLA Adrenergic innervation of cat iris s... 1964 *207* 273—279

TÁKATS I Über den sympathikolytischen Einfluss des Dichlorisoproterenols am isolierten Katzenirissphincter *Albrecht v Graefes Arch Ophthal* 1964 *167* 474—48?

Acta physiol. scand. 1966. 66. 129—132

From the Aero-Medical Laboratory, Institute of Physiology, University of Lund, Sweden

Cardiac Output in the Supine and Sitting Position Determined by a CO_2 Method

By

G Lundin and D Thomson

Received 19 May 1965

Abstract

Lundin, G and D Thomson *Cardiac output in the supine and sitting position determined by a CO_2 method* Acta physiol scand 1966 *66* 129—132 — Cardiac output was determined in the supine and sitting position with a CO_2 rebreathing method. Eight normal adult subjects were used A mean cardiac output of 3 5 l min in the sitting position and 4 3 l min in the supine, was found The stroke volumes were 50 ml and 66 ml respectively

The values for cardiac output in resting recumbent subjects obtained with the CO_2 rebreathing method, described in earlier papers by Jernerus, Lundin and Thomson (1963) and Lundin and Thomson (1965a) were considerably lower than those obtained with the direct Fick or dye dilution methods (Hamilton, 1945) but fairly close to those obtained with the acetylene method (Grollman and Baumann 1935) With the acetylene method the cardiac output determined in the supine was about 0.2 litres higher than in the sitting position Bevegård, Holmgren and Jonsson (1960) using the direct Fick method found in resting subjects a 2 04 l/min higher cardiac output in the supine compared with the sitting position The difficulties in securing normal resting conditions during the direct Fick experiments might be an explanation of not only the large difference between cardiac output supine and sitting but also of the considerably higher values obtained with this method when compared with the indirect values We therefore found it worth while to reinvestigate the problem of cardiac output in the two positions using the CO_2 method in which there is a minimum of physical and psychological disturbance to the subject

Method

The CO_2 content of the mixed venous and arterial blood was indirectly determined as previously described by Lundin and Thomson (1965b and used to calculate the cardiac output using the Fick equation Eight lung heart healthy subjects were used They were not in basal state but were allowed to rest in a comfortable chair in a semireclining position at least 30 min before the

Table I

Subject	Age	Body surface area m²	Supine: Cardiac output l/min Mean	Range	Stroke volume ml Mean	Range
L L.	20	1 89	4 9	4 0—5 6	79	67—93
G L	54	1 98	3 3	3 0—3 5	56	50—58
B R.	21	2 02	4 8	3 5—6 4	74	56—94
W P	24	1 71	5 4	3 8—8 0[1]	81	56—118[1]
D T	25	2 17	6 0	5 4—6 8	83	67—100
U W	28	1 80	4 0	3 0—5 6	59	40—82
H W	20	1 50	2 9	2 5—3 5	41	35—46
H H	22	1 82	3 3	3 0—3 6	58	52—64
Mean		*1 86*	*4 3*		*66*	

[1] These high values were obtained when the subject had an acute upper respiratory infection

actual measurements were started Starts in the sitting position alternated with starts in the supine position Before each measurements there was a resting pause of 10 min in the position to be studied At the end of this period the end tidal CO_2 percentage was measured continously with a rapid infrared CO_2 meter (Godart Capnograph) When a respiratory steady state was [illegible]

[illegible]

Results

Table I gives the mean values from all the experiments The mean cardiac output in the supine position was 4 3 l/min and in the sitting position 3 5, thus giving a difference of 0 8 litres between the two positions Analysis of variance showed this difference to be highly significant In the supine position the mean stroke volume was 66 ml and in the sitting position 50 ml

The CO_2 content of the mixed venous blood was in the supine position 51 7 vol-%, and in the sitting 51 9 vol % This difference of 0 2 vol-% is not significant The CO_2 content of the arterial blood in the supine position was 47 1 vol-% and 46 1 vol % in the sitting, a difference of 1 0 vol % which is significant In the supine subjects the A-V CO_2 difference was 4 6 vol % and in the sitting 5 8 vol %, thus yielding a difference between the two positions of 1 2 vol-%

	Sitting				
	Cardiac output l/min		Stroke volume ml		
Cardiac index l/m²	Mean	Range	Mean	Range	Cardiac index l/m²
2 6	4 4	3 8—4 8	67	59—75	2 3
1 7	3 2	2 7—3 4	51	47—53	1 6
2 4	3 4	3 1—3 6	45	41—47	1 7
3 2	3 1	2 2—4 0	49	37—57	1 8
2 8	4 6	3 1—5 7	62	43—69	2 1
2 2	3 4	2 8—4 5	47	35—61	1 9
1 9	3 0	2 2—3 6	40	31—50	2 0
1 8	2 6	1 9—3 5	41	39—51	1 4
2 3	3 5		50		1 9

Discussion

The difference in cardiac output between the supine and sitting position determined with the CO_2 method is considerably lower than that reported by Bevegård *et al* (1960) with the direct Fick method but higher than the difference found by Grollman and Baumann (1935) with the acetylene method

In a paper by Jernèrus Lundin and Pugh (1963) the solubility of acetylene and its influence on cardiac output was studied with the subject in the sitting position It was found that the solubility of acetylene in the pulmonary tissue caused an error in cardiac output giving 5—10 % too low values This error should be larger in the supine position, because according to Sjostrand (1952) there is a considerable accumulation of blood in the lungs in this position which should increase the amount of acetylene taken up in the lung With the CO_2 method the values for sitting are practically the same as those observed with the acetylene method, thus somewhat too low Lundin and Thomson (1965b) have shown that this does not depend on the CO_2 solubility in lung tissue with the CO_2 method they use However there is according to West (1960) in normal subjects an alveolar-arterial CO_2 pressure gradient of about — 1 mm Hg in the sitting position Provided there is the same difference in CO_2 pressure between the end tidal air and arterial blood this should give about 10 % too low cardiac output values with our method an error of the same magnitude as with the acetylene method In the supine position West found the alveolar-arterial CO_2 pressure gradient to be zero and our cardiac output values in this position should thus not be influenced by the above mentioned error

As discussed above the error of the acetylene method due to the solubility of acetylene in lung tissue should be larger in the supine than in the sitting subjects This means that

the difference in cardiac output found with this method between the two positions is somewhat too low In the CO_2 method on the other hand the difference should be somewhat too high If we therefore assume that we have about 10 % too low cardiac output values in the sitting position with our CO_2 method i e 3 5 l instead of 3 9 l the difference in cardiac output between the two positions is the same as that reported by Donald Bishop and Wade (1953) using the direct Fick method in normal subjects supine and sitting up in bed They found a difference between the two positions of 0 4 litres which might have been somewhat larger if the subjects had been sitting on a chair with their feet on the floor Even when after above mentioned errors for cardiac output values obtained with acetylene or CO_2 methods are taken into consideration there still exist rather large differences between values obtained with indirect methods and values where direct Fick or dye dilution methods have been used for the cardiac output determination with the exception of the values reported by Donald *et al* (1953) We believe that these differences are due to the difficulties in obtaining normal resting conditions with the direct Fick or dye dilution methods The values for cardiac output obtained with methods in which the subjects can be fully relaxed, i e the acetylene or CO_2 methods should therefore better reflect basal circulatory conditions This also means that the difference in cardiac output between the two different body positions is fairly small in most subjects provided that the conditions are basal

References

BEVEGÅRD S A HOLMGREN and B JONSSON The effect of bodyposition on the circulation at rest and during exercise with special reference to the influence on the stroke volume *Acta physiol scand* 1960 *49* 279—298

DONALD K W J M BISHOP and O L WADE The effect of nursing positions on the cardiac output in man *Clin Sci* 1953 *12* 199—216

GROLLMAN A and H BAUMANN *Schlagvolumen und Zeitvolumen* Theodor Steinkopff Dresden 1935

HAMILTON W F Notes on the development of the physiology of cardiac output *Fed Proc* 1945 *4* 183—195

JERNERUS R G LUNDIN and L G C F PUGH Solubility of acetylene in lung tissue as an error in cardiac output determination with the acetylene method *Acta physiol scand* 1963 *59* 1—6

JERNERUS R G LUNDIN and D THOMSON Cardiac output in healthy subjects determined with a CO_2 rebreathing method *Acta physiol scand* 1963 *59* 390—399

LUNDIN G and D THOMSON Mixed venous CO_2 tension determined by a CO_2 rebreathing method Influence of rebreathing time *Acta physiol scand* 1965a *63* 55—57

LUNDIN G and D THOMSON Determination of the mixed venous CO_2 pressure with a rebreathing method Correction for volume changes of the lung bag system *Acta physiol scand* 1965b *64* 448—452

ROOT R N *Handbook of respiration* National Academy of Sciences — National Research Council. W B Saunders Company Philadelphia 1958 64

SJOSTRAND I Regulation of the blood distribution in man *Acta physiol scand* 1957 *26* 312—327

WEST J B and C T DOLLERY Distribution of blood flow and ventilation perfusion ratio in the lung measured with radioactive CO_2 *J appl Physiol* 1960 *15* 405—410

Acta physiol. scand. 1966. 66. 133—145

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Two Types of Stretch-Activated Post-Synaptic Inhibitions in Spinal Motoneurones as Differentiated by Strychnine

By

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Abstract

[illegible]

types of inhibition were shown to act on the post synaptic membrane. Strychnine resistant post synaptic inhibition was frequently encountered and was found to have characteristics similar to those described for presynaptic inhibition.

Based on the results of studies using micro-electrode recording technique as well as other methods, two kinds of inhibitory mechanisms in the spinal cord have been postulated (Eccles, 1964)

a) post-synaptic inhibition, where the post synaptic membrane potential through hyperpolarization is moved away from the threshold level at which nerve impulses are initiated, and

b) presynaptic inhibition, which depolarizes the presynaptic afferent terminals and thereby diminishes the afferent spike amplitude leading to a diminution of the amount of transmitter substance released by the arriving nerve impulses.

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These conclusions have mainly been drawn from work where the afferent inflow has been induced by electrical stimulation of the peripheral nerves When the effects of these synchronous volleys are recorded intracellularly, the following two events have been used as criteria for inhibition

i) *hyperpolarization* of the post-synaptic membrane, and/or

ii) *decreased monosynaptic excitability* as measured by a diminution of the excitatory post synaptic potential (EPSP)

As shown by Granit, Kellerth and Williams (1964*a*,*b*), however, these two indicators for inhibition were not always valid when muscle stretch was used for stimulation Granit *et al* (1964*b*) instead introduced two other criteria for identifying inhibition which were found to be more reliable, namely

iii) *reduction of the firing rate* of a neurone stimulated through the intracellular electrode by injected depolarizing current and,

iv) *synaptic activation noise'* which during inhibitory activity should be in the hyper polarizing direction

When the latter two criteria were satisfied, this was held to be definite evidence for inhibitory action on the post synaptic membrane By using these criteria Granit *et al* (1964*b*) in no case found it necessary to explain the effects of maintained stretch on the basis of presynaptic events

Pharmacological investigations (Curtis, 1959, Eccles, Schmidt and Willis, 1963) indicate that pre and post-synaptic inhibitions differ in their response to certain convulsive agents *strychnine* was found to abolish post synaptic inhibition, but left the presynaptic inhibitory component intact or even slightly enhanced, *picrotoxin*, on the other hand, was found not to affect the post-synaptic inhibition, but to reduce the presynaptic inhibitory action

In this paper the effects of strychnine on inhibitions of motoneurones stimulated by muscle stretch will be described Special attention will be paid to the different criteria mentioned above A brief preliminary report has already been published (Kellerth 1965)

The effects of picrotoxin will be presented separately in a subsequent paper (Kellerth and Szumski, 1965)

Methods

Cats were anaesthetized with pentobarbitone 35 mg/kg Rigid fixation of the animal was obtained by clamping at the vertebral column iliac crest and ankles while the greater trochanter and lateral condyle of the left femur were screwed to fixed supports

The triceps surae tibialis anterior and semitendinosus muscles of the left hind limb were [illegible]

amplifier was also used to monitor the activity of the neurone

Stimulating electrodes were placed on the intact popliteal common peroneal and hamstring nerves and on the cut proximal segment of ventral roots L7 + SI Popliteal peroneal and hamstring motoneurones were identified by their monosynaptic response Autogenetic effects were represented in popliteal motoneurones by stretch of triceps surae in hamstring motoneurones by semitendinosus stretch and in peroneal motoneurones by tibialis anterior stretch. Essentially this is the technique used by Granit *et al* (1964*a*)

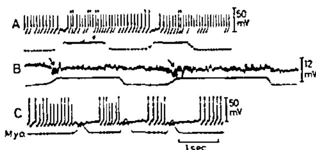


Fig 1 Popliteal motoneurone of 74 mV spike height *A* The neurone was fired by injected current Stretches (500 g) of tibialis anterior, as indicated by upward deflexions of the myograph caused phasic inhibitions of the discharge *B* Higher d c sensitivity was used to show the effects from stretches (500 g) of tibialis anterior on the average membrane potential *C* was recorded after an i v injection of succinylcholine (0 8 mg/kg) The neurone was fired by injected current and brief manual stretches of tibialis anterior now caused long lasting inhibitions

Micro-electrodes filled with 2 M potassium citrate (4—10 MΩ resistance) were used for intracellular recording and stimulation The bridge circuit for stimulation was similar to that of Araki and Otani (1955)

Strychnine nitrate was administered i v at various dosages as indicated in the text up to a maximum of 0 2 mg/kg Gallamine triethiodide (Flaxedil) 20 mg/kg was given prior to strychnine to eliminate overt convulsive activity The animal was respired on a pump and generally a bilateral pneumothorax was performed

Results

The results to be described in this paper have been collected from 54 motoneurones ranging in spike height from 50—98 mV Motoneurones were selected which were able to respond with a maintained discharge of impulses of a regular frequency during a long lasting (15—40 sec) injection of depolarizing current Only neurones in good condition responded on this manner

The standard procedure of recording was to start with the effect of muscle stretch on repetitive firing induced by means of injected depolarizing current, since a back

simultaneous shifts of the average level of the membrane potential The effect of muscle stretch upon the size of the monosynaptic EPSP was often but not always recorded mainly because this criterion had been found to be an equivocal index of inhibition (Granit *et al* 1964*b*) The procedure of recording is exemplified in Fig 7

This paper will be divided into two parts Part I will include examples of post synaptic inhibitions which as expected from previous work in this field were abolished after the administration of strychnine Part II will include examples of strychnine resistant post synaptic inhibitions

Part I

Figures 1 and 2 refer to an impaled popliteal motoneurone of 74 mV monosynaptic spike size In Fig 1 *A* the neurone was fired repetitively by injected depolarizing current When the tibialis anterior was stretched with a 500 g weight (upward deflection of the myograph) there occurred a phasic inhibition of the repetitive discharge

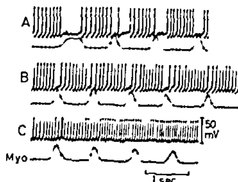


Fig 2 Same motoneurone as in Fig 1, after the administration of 0 2 mg/kg of strychnine The neurone was fired by injected current Brief stretches were applied to tibialis anterior *A* 10 sec, *B* 15 sec and *C* 25 sec after strychnine was injected The inhibition was completely removed by strychnine (compare Fig 1 *C*)

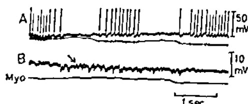


Fig 3 Popliteal motoneurone of 55 mV spike height *A* The neurone was fired by injected current and was inhibited by stretches (200 g) of semitendinosus *B* Higher d c sensitivity was used to show the hyperpolarizing activation noise during stretch (200 g) of semitendinosus, especially prominent at arrow

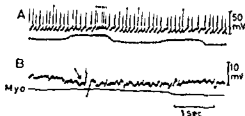
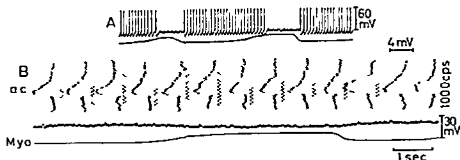


Fig 4 Same motoneurone as in Fig 3 after the administration of 0 12 mg kg of strychnine *A* The neurone was fired by injected current No inhibitions are now seen from stretches (200 g) of semitendinosus *B* Higher d c sensitivity shows that the hyperpolarizing activation noise had been removed by strychnine (compare Fig 3) Arrow indicates an action potential The drift of base line during muscle stretch in *B* was caused by mechanical movement of the cord

In the d c recording at a higher sensitivity in *B*, this inhibitory activity is seen to give rise to a hyperpolarizing shift of the average level of the membrane potential (arrows) of the order of 4 mV The wide and irregular base line during these short lasting hyperpolarizations is caused by the presence of 'synaptic activation noise'

The inhibition recorded above was caused by an antagonistic muscle, and judging from its phasic character it could possibly be mediated by Ia afferents In order to verify this assumption an injection of succinylcholine (0 8 mg/kg i v) was given This drug has the property of sensitizing the muscle spindles (Granit Skoglund and Thesleff 1953) with some preference for the Ia afferents (Verhey and Voorhoeve 1963) The effect of succinylcholine is shown in *C* where now long lasting inhibitions were evoked by brief stretches of the tibialis anterior (compare with *A* in the same Fig)

While still recording inside the same motoneurone strychnine was given i v in a dose of 0 2 mg/kg In Fig 2 *A* is seen the effect of brief stretches of the tibialis anterior



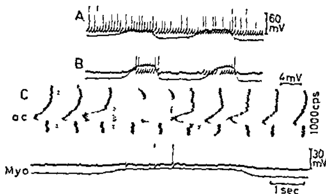
10 sec after the administration of strychnine (to be compared with Fig 1 *C*) Figure 2 *B* was recorded 5 sec later In 2 *C*, 25 sec after strychnine was given, this inhibition was completely removed Thus, this particular inhibition was found to be post synaptic as verified by all three identifying criteria The effect of stretch upon the size of the EPSP was not studied Since the effect of this strychnine sensitive inhibition was considerably enhanced by succinylcholine, it is again concluded that it arose from the discharge of stretch receptors over Ia afferents

Figures 3 and 4 illustrate another popliteal motoneurone (spike size 55 mV) which was fired repetitively by means of injected current (Fig 3 *A*) A 200 g stretch of semitendinosus was here sufficient to inhibit the discharge. In 3 *B* a higher d c -gain was used to show the ' activation noise" that occurred during the same amount of stretch This noise was in a purely hyperpolarizing direction There was, however, no shift of the average level of the membrane potential and yet, as shown by the criteria used in 3 *A* and *B* the inhibition was clearly post-synaptic

Figure 4 illustrates the behaviour of the same motoneurone after the administration of 0 12 mg/kg of strychnine As seen in *A* the inhibitory influence from semitendinosus had been removed, and in *B* is seen that the hyperpolarizing "noise" had been replaced by dominant excitatory activity (compare with Fig 3 *B*)

Finally, Figs 5 and 6 illustrate another popliteal motoneurone of 62 mV monosynaptic spike height Figure 5 *A* shows that a 500 g stretch of semitendinosus inhibited the discharge initiated by injected depolarizing current In 5 *B*, the continuous d c.-recording of the average level of the membrane potential reveals that a hyperpolarizing shift of the order of 4 mV took place during the stretch Simultaneously, there was a diminution of the size of the EPSP as shown in the fast sweeps Several repeated *recordings averaged the size of the control EPSP to 3 3 mV, and the EPSP during* muscle stretch to 2 8 mV, a depression of 15 % In this motoneurone synaptic activation noise was the only criterion that failed to show inhibition.

While the micro-electrode was still impaled in this motoneurone, 0 08 mg/kg of strychnine was injected The effect is shown in Fig 6 A where now no inhibition during muscle stretch remained Another 0 05 mg/kg strychnine was then given In 6 *B* a current of subthreshold strength was injected into the motoneurone and now



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monosynaptic excitability (a c sweeps) and the average membrane potential (continuous d c recording) Occasional monosynaptic spikes are seen to fire on stretch

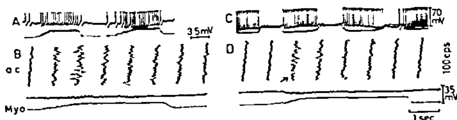
stretch of the muscle fired the neurone This response demonstrates so-called strychnine reversal (compare with Fig 5 A)

As seen in the continuous d c recording of Fig 6 C the former hyperpolarization during muscle stretch had turned into a depolarization which was of the order of 2 5 mV Also the monosynaptic test response (a c sweeps in 6 C) reversed its former behaviour and was now increased by stretch from 2 0 mV to 2 7 mV (+ 35 %) Occasionally it included monosynaptic spikes This marked increase in size of the EPSP during stretch, despite the simultaneous depolarization of the membrane, might be unexpected One possible explanation is that the excitation which appeared after the administration of strychnine actually was a 'disinhibition of the motoneurone Such a disinhibition might have fired the motoneurone (Fig 6 B) and may also have been responsible for the depolarization seen in Fig 6 C The absence of synaptic activation noise' also supports this view Since disinhibition ' consists of a removal of tonic synaptic activity it would cause a decreased conductance in the motoneurone membrane and hence produce the increase in voltage change (EPSP) that was seen during stretch in response to a stimulus of constant strength

This explanation would then imply the existence of a type of strychnine resistant interneuronal chain activated by peripheral receptors which has not yet been described As will be shown in Part II of this paper however these strychnine resistant inhibitory mechanisms may even act directly upon the post synaptic membrane of motoneurones

Part II

In this section will be described motoneurones which were either recorded from already heavily strychninized cats and nevertheless showed strong inhibitory effects or else were penetrated in non strychninized cats and found to have their post synaptic inhibitions intact after the administration of strychnine



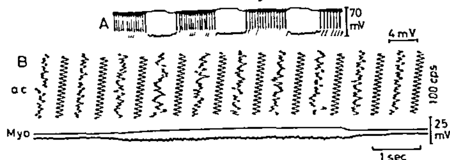


Fig 8 Strychninized cat (0 2 mg kg) Popliteal motoneurone of 67 mV spike height *A* The neurone was fired by injected current The discharge was inhibited by stretch (200 g) of semitendinosus *B* Stretch (200 g) of semitendinosus caused hyperpolarizing "activation noise (a c sweeps and a hyperpolarizing shift of the average membrane potential (continuous d c recording

Figure 7 shows a popliteal motoneurone of 73 mV monosynaptic spike size Ten minutes before this neurone was impaled the cat had been given 0.2 mg/kg of strychnine, which is sufficient to abolish essentially all strychnine-sensitive inhibitions (Bradley Easton and Eccles 1953) In Fig 7 *A* a subthreshold depolarizing current was injected When applying a 500 g stretch to the triceps surae the neurone was excited to fire as would be expected from what is known about autogenetic stretch This excitation caused large depolarizing wavelets of synaptic activation noise' (in *B* on the fast a c sweeps) which corresponded to a 2 mV depolarizing shift of the average level of the membrane potential In *C* induced repetitive firing was inhibited by 500 g stretch of the tibialis anterior The post-synaptic character of this inhibition is confirmed by the hyperpolarizing activation noise' (especially prominent at arrow in *D*) which occurred simultaneously with a hyperpolarizing shift of the average level of the

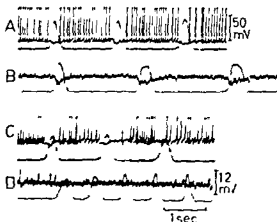


Fig 9 Popliteal motoneurone of 84 mV spike height A and B were recorded before strychnine was given C and D after the administration of 0 03 mg/kg of strychnine A The neurone was fired by injected current Brief stretches (500 g) of tibialis anterior inhibited the discharge B Higher d c -sensitivity shows the hyperpolarizing shifts of the membrane potential caused by brief stretches (500 g) of tibialis anterior C The neurone was fired by injected current The inhibition from stretch (500 g) of tibialis anterior was still seen after strychnine D No shift of average membrane potential was now seen during stretch of tibialis anterior The hyperpolarization seen in B had been removed by strychnine

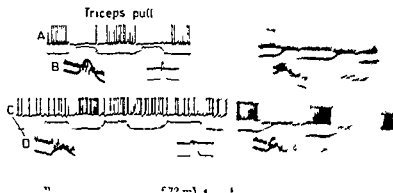
membrane potential of the order of 3 5 mV (continuous d c -recording of D) This neurone also received strong post-synaptic inhibition from semitendinosus (not shown in this Figure)

Thus despite the heavy strychninization, this extensor neurone received powerful inhibition from two antagonistic flexor muscles, and by the three criteria utilized here these inhibitions proved to be of post synaptic origin In this case the effect of muscle stretch upon the size of the monosynaptic EPSP was not studied

Fig 8 illustrates another experiment in which the cell was impaled 12 min after 0 2 mg/kg of strychnine had been given to the animal It is a popliteal motoneurone of 67 mV spike size The cell was fired repetitively by injected current in A, and when a 200 g stretch was applied to the semitendinosus, a powerful inhibition wholly abolished the discharge In the fast sweeps of B is seen the hyperpolarizing activation noise" during the stretch showing the post-synaptic nature of this inhibition, while the standing d c -spot at the same time recorded a hyperpolarization of the membrane of the order of 2 4 mV The size of the monosynaptic EPSP of this neurone was reduced during stretch from 2 8 mV to 2 5 mV (11 % diminution)

An interesting experiment is illustrated in Fig 9, which suggests that different inhibitory mechanisms arising from the same stretch can act simultaneously upon the same post-synaptic membrane This was a popliteal motoneurone of 84 mV spike size, which was impaled in a non strychninized cat In A the neurone was fired repetitively by injected depolarizing current When the tibialis anterior was stretched with 500 g the discharge is seen to have been inhibited Recording with high d c gain (B) demonstrates that the stretch caused a hyperpolarization of the membrane of the order of 3 mV Activation noise' during stretch was also heard in the loudspeaker

Between B and C strychnine was injected in a dose of 0 03 mg/kg, and the recording procedure was repeated As seen in C the inhibitory activity was still present and not even reduced A most interesting effect is seen in D where now the hyperpolarization during stretch has disappeared compare with B) Weak but clearly distinguishable "activation noise" was, however, still recorded during stretch, proving that there was a



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These results clearly pro e that t o types

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TABLE I Summary of motoneurones studied after the administration of strychnine

Muscle causing inhibition	Neurone receiving inhibition		
	Hamstring	Popliteal	Common Peroneal
Semitendinosus	1	11	1
Triceps surae	1	—	2
Tibialis anterior	5	13	2
	Neurones receiving no inhibition		
	8	10	5

activation noise" in a hyperpolarizing direction Brief stretches of triceps surae caused an initial short lasting hyperpolarizing shift of the average membrane potential of the order of 2 mV When similarly stretching tibials anterior, the membrane was hyperpolarized by 3 mV, and the duration of this potential shift was as long as 600 msec It thus considerably outlasted the duration of the stretch which was about 300 msec

Strychnine was then injected in a dose of 0 08 mg/kg, and after one minute the experiment was repeated It is now seen that the inhibitory effect from triceps surae had been largely removed (*C*) that its activation noise" exhibited depolarizing wavelets and that a depolarizing shift of the membrane potential of 1 5 mV (*D*) had replaced the previous hyperpolarization The inhibition from tibialis anterior, on the contrary, had been markedly potentiated (*C*) after strychnine The duration of the hyperpolarization (*D*), which was still of the order of 3 mV, had now been extended in duration to around 900 msec A definite increase of synaptic activation noise" was not recorded

This experiment reveals the existence of two types of post synaptic inhibitory mechanisms which may act upon the same motoneurone, one abolished or decreased by strychnine, the other unaffected or even potentiated

Table I summarizes the results from 54 motoneurones which were investigated in this study As seen from the table, about 50 % of the flexor (hamstring and common peroneal) motoneurones received strychnine resistant post synaptic inhibition from flexor or extensor muscles while 70 % of the extensor (popliteal) motoneurones showed inhibition which was initiated only from flexor muscles This indicates a tendency for strychnine-resistant post synaptic inhibition preferably to originate from flexor muscle afferents and act on the extensor Ia reflex arc, a pathway which has also been described as important for presynaptic inhibition The rather high percentage of tibialis anterior inhibitions acting on hamstring motoneurones may seem unexpected but can be explained by the fact that many semitendinosus motoneurones act as synergists to triceps surae (Granit *et al* 1964*a*) Another striking feature is that although most of these motoneurones belong to the popliteal population, we have never been able to find an autogenetic inhibition from triceps surae that was resistant to strychnine (cf e g Fig 10)

Discussion

In part I of this paper were given examples of post synaptic inhibitions on spinal motoneurones, the effects of which were abolished after the administration of strychnine Until now this response to strychnine has been believed to be true for all segmental post synaptic inhibitions in the spinal cord of the cat (Bradley *et al* 1953, Curtis, 1959, 1962, 1963)

Part I of the present paper also confirms the significance of hyperpolarizing activation noise" in revealing post synaptic inhibitory influences (Granit *et al* 1964*a*) by showing that in strychnine-sensitive inhibitions hyperpolarizing 'activation noise" was removed by the drug (see Fig 2 and 3)

In Part II of this paper were presented further studies of the type of strychnine-resistant post synaptic inhibitory mechanism in motoneurones activated by peripheral receptors, that was first described by Kellerth (1965) This type of inhibition frequently occurred among the spinal motoneurones investigated Its properties may be summarized as follows a) It is resistant to strychnine, or b) even potentiated by strychnine (see e g Fig 10), and c) is preferably derived from flexor muscle afferents acting upon the post synaptic membrane of extensor motoneurones (see Table 1) The effects of cutaneous afferents have not been studied

All three properties listed above have also been described as characteristic for presynaptic inhibition, one important pathway of which is described to act on the terminals of the extensor Ia afferents which project onto their own motoneurones

Another property of presynaptic inhibition is its sensitivity to picrotoxin (Eccles *et al* 1963), and in a subsequent paper (Kellerth and Szumski 1965) will be described the effects of picrotoxin upon strychnine resistant post synaptic inhibition

Strychnine resistant post synaptic inhibitions have earlier been described in hippocampus

descending inhibition from the reticular formation on lumbar extensor motoneurones is removed only by a combination of strychnine and mephenesin while picrotoxin on the other hand is ineffective This result may well indicate the existence of a third type of post-synaptic inhibitory mechanism acting on spinal motoneurones

Since in the past many investigators were concerned with the inhibitions from peripheral receptors on motoneurones it may seem surprising that the strychnine resistant post synaptic inhibition has remained undiscovered until now Possible reasons for this may be a) Many earlier investigations have dealt with the inhibition of the monosynaptic reflex as recorded from the ventral roots which must be assumed to give only a rather rough estimation of the effects of strychnine upon the activity of spinal neurones b) In studies with intracellular recording technique on the other hand, the criteria used to identify inhibition have been hyperpolarization of the post synaptic membrane and/or decreased monosynaptic test responses (criteria I and II in the Introduction) However Granit *et al* (1964*b*) have shown that, at least with muscle stretch as a stimulus these criteria for inhibition are not as reliable as the combination of all four criteria that have been used in this study (see Introduction), c) There is further the possibility that the strychnine resistant post-synaptic inhibition might

exert its main action in places remote to the soma, where the electrode tip is supposed to be situated, and hence produce post synaptic inhibitory effects, which the electrode would not be able to detect (Frank 1959) However, this view receives no support from our results because both types of post-synaptic inhibitions produced hyperpolarizing 'activation noise' and hyperpolarizing shifts of the average level of the membrane potential, d) Most previous work has been concerned with post-synaptic inhibitions arriving along short latency pathways, while the long polysynaptic pathways have been less studied In the work by Curtis (1962), in which strychnine was applied electrophoretically, longer polysynaptic pathways were also investigated, and here were found IPSP's that were not affected by the ejection of strychnine This effect was ascribed to inability of the strychnine to reach the synapses To judge by our results these IPSP's might well have been strychnine resistant e) Furthermore, it is hardly justified to assume that an entire IPSP is sensitive to strychnine because its amplitude is reduced by the drug Our study has clearly shown that both types of post-synaptic inhibition can act simultaneously upon the same membrane

The early observations (Liddell and Sherrington 1925, Cooper and Creed 1927) to the effect that strychnine did not reverse the inhibitions exerted on quadriceps stretch reflexes by pulling on the hamstring muscle, have been interpreted as a clear case of presynaptic inhibition (Eccles 1965) *The present results demonstrate that this inhibition can be satisfactorily explained by post synaptic events* Whether or not presynaptic inhibition also plays a role in the maintained stretch reflexes studied above is at the moment unknown

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References

ANDERSEN P J C ECCLES Y LØYNING and P E VOORHOEVE Strychnine resistant inhibition in the brain *Nature* (Lond 1963 *200* 843—845

ARAKI, T and T OTANI Response of single motoneurones to direct stimulation in toad's spinal cord *J Neurophysiol* 1955 *18* 472—485

BAUMGARTEN R von J D GREEN and M MANCIA Recurrent inhibition in the olfactory bulb II Effects of antidromic stimulation of commissural fibers *J Neurophysiol* 1962 *25* 489—500

BRADLEY K D M EASTON and J C ECCLES An investigation of primary or direct inhibition. *J Physiol* (Lond 1953 *122* 474—488

BREMER F Recherches sur le mechanism de l action de la strychnine sur le système nerveux. *Arch int Physiol* 1925 *25* 131—152

COOPER, S and R S CREED More reflex effects of active muscular contraction *J Physiol* (Lond) 1927 *64* 199 214

CRAWFORD J M D R CURTIS P E VOORHOEVE and V J WILSON Strychnine and cortical inhibition. *Nature* Lond 1963 *200* 845—846

CURTIS D R Pharmacological investigations upon inhibition of spinal neurones *J Physiol* (Lond) 1959 *145* 175 192

CURTIS D R The depression of spinal inhibition by electrophoretically administered strychnine *Int J Neuropharmacol* 1962 *1* 239 250

CURTIS D R The pharmacology of central and peripheral inhibition *Pharmacol Rev* 1963 *15* 333—364

ECCLES, J C., *The physiology of synapses* Springer Verlag Berlin Göttingen Heidelberg 1964

ECCLES, J C. Pharmacology of central inhibitory synapses *Brit med Bull* 1965 *21* 19—25

ECCLES J C., R F SCHMIDT and W D WILLIS Pharmacological studies on presynaptic inhibition *J Physiol* (Lond) 1963 *168* 500—530

FRANK K Basic mechanisms of synaptic transmissions in the central nervous system *IRE Trans Med Electronics ME 6* 1959 85—88

GRANIT, R J O KELLERTH and T D WILLIAMS Intracellular aspects of stimulating motoneurones by muscle stretch *J Physiol* (Lond) 1964*a* *174* 435—452

GRANIT R J O KELLERTH and T D WILLIAMS 'Adjacent' and 'remote' post synaptic inhibition in motoneurones stimulated by muscle stretch *J Physiol* (Lond) 1964*b* *174* 453—472

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25 467—488

KELLERTH J O A strychnine resistant postsynaptic inhibition in the spinal cord *Acta physiol scand* 1965 *63* 469—471

KELLERTH J O and A J SZUMSKI Effects of picrotoxin on stretch activated post synaptic inhibitions in spinal motoneurones *Acta physiol scand* 1966 *66* 146—156

LIDDELL, E G T and C S SHERRINGTON Further observations on myotatic reflexes *Proc roy Soc* B 1925 *97* 267—283

LLINAS R Mechanisms of supraspinal actions upon spinal cord activities Differences between reticular and cerebellar inhibitory actions upon alpha extensor motoneurones *J Neurophysiol* 1964*a* *27* 1117—1126

LLINAS R Mechanisms of supraspinal actions upon spinal cord activities Pharmacological studies on reticular inhibition of alpha extensor motoneurones *J Neurophysiol* 1964*b* *27* 1127—1137

MAGNUS R and C F WOLF Weitere Mitteilungen uber den Einfluss der Kopfstellung auf den Gliedertonus *Pflug Arch ges Physiol* 1913 *149* 447—461

Acta physiol scand 1966 66 146—156

From the Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden

Effects of Picrotoxin on Stretch-Activated Post-Synaptic Inhibitions in Spinal Motoneurones

By

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Abstract

Kellerth J O and A J Szumski *Effects of picrotoxin on stretch activated post synaptic inhibition in spinal motoneurones* Acta physiol scand 1966 66 146—156 — The effects of picrotoxin on post synaptic inhibitions were intracellularly investigated in cat popliteal common peroneal and hamstring motoneurones which were activated by stretch of the triceps surae tibialis anterior or semitendinosus muscles In some experiments a single electrical shock to the nerve was also used as a stimulus Inhibitions from muscle stretch were studied by the following criteria [illegible]

[illegible]

chloride ions Post synaptic inhibitions remaining after picrotoxin (picrotoxin resistant) and those abolished by picrotoxin (picrotoxin sensitive) were identified Both types of inhibition were shown definitely to act on the post synaptic membrane As with strychnine resistant post synaptic inhibition (Kellerth and Szumski 1966) picrotoxin sensitive post synaptic inhibition was found to have characteristics similar to those described for presynaptic inhibition In no case was a post synaptic inhibition found to be resistant to both strychnine and picrotoxin and in strychninized cats the remaining post synaptic inhibitions were always abolished by picrotoxin

In a previous paper the existence of a hitherto unknown type of stretch activated post synaptic inhibition in cat lumbar motoneurones was described (Kellerth and Szumski 1966) This post synaptic inhibitory mechanism which was found frequently to occur was insensitive to strychnine or even potentiated by the drug and was found to have an important pathway in flexor muscle afferents acting on extensor motoneurones Since these properties also have been described as being characteristic for presynaptic inhibition (Eccles 1964) it was considered to be of interest to study the effects of picro-

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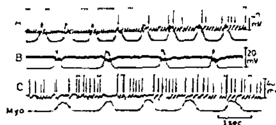


Fig 1 Non strychninized cat. Hamstring motoneurone of 65 mV spike height *A* Neurone fired by injected depolarizing current and brief manual stretches of the triceps surae muscle (upward deflections of the myograph) caused phasic inhibition of the discharge *B* High d c sensitivity revealed hyperpolarizing shift of average membrane potential of 4 0 mV during muscle stretch *C* Recorded after i v injection of picrotoxin (1 5 mg/kg) Even though convulsive firing occurred the inhibitory influence to stretch is still present

toxin on the strychnine resistant post synaptic inhibitions. According to current views, picrotoxin blocks presynaptic inhibition (Eccles Schmidt and Willis 1963) but has no effect on post synaptic inhibition The present study shows that the effects of picrotoxin on inhibitory mechanisms in the spinal cord are more complex

Methods

The experimental approach was identical to that described in a previous paper (Kellerth and Szumski 1966) except for a number of cats (indicated in the text) which were spinalized at cord segment L2

Micro-electrodes filled with 2 M potassium citrate (4—10 MΩ resistance) were used for

tively

Results

The present results were obtained from twenty two motoneurones seven of which impaled in non strychninized cats Of the fifteen motoneurones impaled in strychninized cats nine originate from spinalized cats The criteria used to identify inhibitions have been described in a previous pharmacological study (Kellerth and Szumski 1966) and are those used by Granit Kellerth and Williams (1964 b) Briefly these criteria are as follows i) hyperpolarization of the post-synaptic membrane ii) decreased monosynaptic excitability as measured by a diminution of the excitatory post synaptic potential iii) reduction of firing rate of a neurone stimulated through the intracellular micro-electrode by injected depolarizing current and iv) synaptic activation noise in a hyperpolarizing direction

Fig 1 illustrates a hamstring motoneurone of 65 mV monosynaptic spike height which was impaled in a non strychninized cat In *A* the neurone was fired repetitively by injected depolarizing current and the discharge is seen to be inhibited by brief manual stretches of the triceps surae muscle (upward deflections of the myograph In *B* increased d c gain reveals that a 4 0 mV hyperpolarizing shift of the average level of the membrane potential took place during stretch of triceps surae The muscle stretch

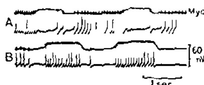


Fig 2 Strychninized cat (0 2 mg/kg) Hamstring

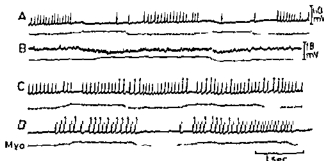
During stimulation of neurone with just subthreshold injected depolarizing current stretches (500 g) of triceps surae now excited it to fire

also caused a 10 % diminution of the size of the monosynaptic test response (not shown in the Fig) Synaptic activation noise" was heard from the loudspeaker, indicating activity at the post synaptic membrane Picrotoxin was then injected i v in a dose of 1 5 mg/kg In *C* the neurone began convulsive firing, but the inhibitory influence from stretch of triceps surae was still present Thus in accordance with current views (Eccles 1964) this post synaptic inhibition was found to be unaffected by picrotoxin

In subsequent results to be presented in this paper, only inhibitions sensitive to picrotoxin will be described

Fig 2 is a record of a hamstring motoneurone of 60 mV spike height which was impaled in a *strychninized (0 2 mg/kg) cat In A stretches (500 g) of triceps surae* inhibited the discharge caused by injected depolarizing current No shift of the average level of the membrane potential was detected during muscle stretch but hyperpolarizing synaptic activation noise' was recorded showing inhibitory activity on the post synaptic membrane Picrotoxin was then injected i v in a dose of 1 0 mg/kg and the results in *B* were recorded A just subthreshold depolarizing current was here injected and now stretch (500 g) of triceps surae excited the neurone to fire, showing that the action of muscle stretch was reversed after the administration of picrotoxin

Another experiment from a strychninized (0 2 mg/kg) cat is illustrated in Fig 3 where a peroneal motoneurone (70 mV spike height) was fired by injected depolarizing current In *A* the repetitive discharge is seen to be inhibited by stretch (500 g) of triceps surae In *B* the same stretch caused a 3 5 mV hyperpolarizing shift of the average



(70 mV spike height *A* Neurone inhibition of discharge the average membrane ...otoxin ... m n ... ected (2 0 mg/kg) after the inje... depolarizing current stretch of triceps surae now excited it to fire

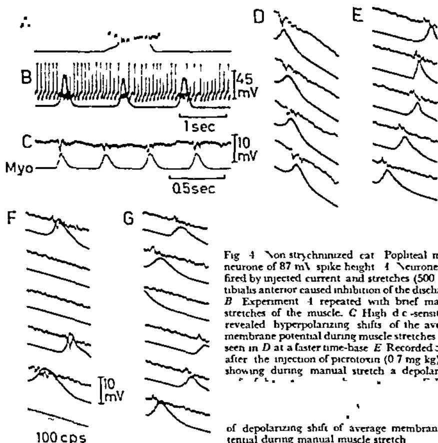


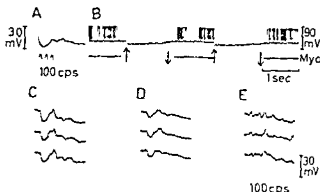
Fig 4 Non strychninized cat Popliteal motoneurone of 87 mV spike height A Neurone was fired by injected current and stretches (500 g) of tibialis anterior caused inhibition of the discharge B Experiment A repeated with brief manual stretches of the muscle. C High d c -sensitivity revealed hyperpolarizing shifts of the average membrane potential during muscle stretches also seen in D at a faster time-base E Recorded 5 min after the injection of picrotoxin (0 7 mg kg) and showing during manual stretch a depolarizing [illegible]

of depolarizing shift of average membrane potential during manual muscle stretch

level of the membrane potential Synaptic activation noise' of mixed character but with clearly hyperpolarizing components was present muscle stretch indicating the post-synaptic character of this strychnine-resistant inhibition

Picrotoxin was then administered i v in a dose of 2 0 mg/kg After 1 5 min a depolarizing current was injected to fire the neurone repetitively (C), and stretch (500 g) of triceps surae now caused only a slight amount of inhibition After another 0 5 min a subthreshold depolarizing current was injected (D) and the muscle stretch now excited the neurone to fire repetitive action potentials, another example of a strychnine resistant post synaptic inhibition which reversed to excitation after the administration of picrotoxin

Fig 4 illustrates a popliteal motoneurone of 87 mV spike height which was recorded from a non-strychninized cat The neurone was fired repetitively by injected depolarizing current and in A the discharge was completely inhibited by a maintained stretch (500 g of tibialis anterior Briefer manual stretches of the muscle B) also inhibited the repetitive activity In C using high d c gain are shown the hyperpolarizing shifts of the average level of the membrane potential of approximately 3 0 mV which



brief tetanic stimulus (4 shocks at 600 sec) to the common peroneal nerve *D* Inhibitory potential in *C* was reduced 2 min after injection of picrotoxin (0 7 mg/kg) *E* Six min after picrotoxin inhibitory potential was completely removed

occurred during brief manual stretches of the muscle In *D* a faster time base was used to illustrate the same response as in *C* Synaptic activation noise ' in the hyperpolarizing direction was also recorded during stretch of the muscle (not shown in the Fig) indicating the post synaptic nature of this inhibition

Picrotoxin was then injected in a dose of 0 7 mg/kg After 5 min the manual stretches of the tibialis anterior muscle (*E*) reversed former effect on the motoneurone membrane and caused a depolarizing shift of the average level of the membrane potential of around 2 0 mV (compare with D of the same Fig) In this particular experiment the administered dose of picrotoxin was apparently not sufficient to maintain the reversed behaviour of the neurone during muscle stretch because in *F* 8 min after the injection the hyperpolarizing response returned A second dose of picrotoxin (0 45 mg/kg) was then added and 30 sec later (*G*) this post synaptic inhibition again underwent a picrotoxin reversal

Fig 5 illustrates a hamstring motoneurone of 74 mV spike height in a non strychninized cat The membrane potential amounted to 65 mV (measured as the potential difference between inside and outside) Record *A* shows the large afterhyperpolarization of 9 5 mV following an antidromic action potential It was not possible to alter the excitability of this neurone by muscle stretch but on the other hand the neurone showed large inhibitory post synaptic potentials when the common peroneal nerve was electrically stimulated In *B* the neurone was fired repetitively by injected depolarizing current Maintained tetanic stimulation (indicated by arrows) of the common peroneal nerve caused a powerful inhibition of the discharge In *C* is seen the inhibitory post synaptic potential produced by a brief tetanic stimulus (4 shocks at 600/sec) to the common peroneal nerve The latency of this inhibition exceeded that of the monosynaptic spike by 3 5 msec The maximum hyperpolarization which amounted to around 13 0 mV was reached 7—8 msec after its onset and its total duration was of the order of 25 msec

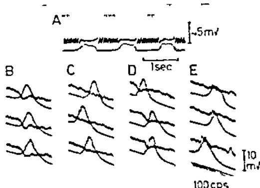


Fig 6 Non strychninized cat Popliteal motoneurone of 73 mV spike height impaled with a KCl *micro-electrode* (see text) *A* Neurone fired by injected current and manual stretches of the tibialis anterior muscle caused an inhibition of the discharge *B* High d c -sensitivity revealed a hyperpolarizing shift of the average membrane potential during brief manual stretches of tibialis anterior *C* After injecting chloride ions into the neurone no hyperpolarization of the post-synaptic membrane was recorded during muscle stretch *D* One min after chloride ion injection was discontinued hyperpolarization of the post synaptic membrane reappeared during muscle stretch *E* Three min after injection of picrotoxin (0 5 mg/kg) the average membrane potential during muscle stretch was in the depolarizing direction

While still recording inside this motoneurone, picrotoxin was administered i v in a dose of 0 7 mg/kg In *D*, 2 min after the injection of picrotoxin, the size of this hyperpolarizing potential was reduced Six min after picrotoxin was given (*E*), the inhibitory *post-synaptic potential was completely removed by the drug*

Fig 6 is a record of an experiment where a KCl micro-electrode was used, in order to study the effects of excess Cl-ions on picrotoxin-sensitive post synaptic inhibition (Coombs, Eccles and Fatt 1955, Eccles, Eccles and Ito 1964) The impaled neurone was a popliteal motoneurone of 73 mV spike height in a non strychninized cat, whose repetitive discharge induced by injected depolarizing current, was inhibited by manual stretches of the muscle tibialis anterior However, no definite synaptic activation noise" was recorded in response to muscle stretch

Chloride ions were then injected into the motoneurone by applying a hyperpolarizing current (30 nA) through the micro-electrode for 70 sec As seen in *C*, recorded immediately *after the chloride ion injection, no hyperpolarization of the post-synaptic membrane* could then be detected during muscle stretch This result defines the inhibition as post synaptic in accordance with the studies by Coombs *et al* (1955), and Eccles *et al* (1964) One min after the injection of chloride ions (*D*), however, the neurone recovered and the hyperpolarization of the post synaptic membrane during muscle stretch reappeared Picrotoxin was then administered i v in a dose of 0 5 mg/kg Three min later (*E*) the muscle stretch reversed its former action on the post-synaptic membrane and now caused a 2 0 mV depolarizing shift of the average level of the membrane potential In summary the post-synaptic nature of the inhibition was in this case demonstrated by one more criterion, namely its sensitivity to an increased *intracellular concentration of chloride ions* (Coombs *et al* 1955) *which thereby* was found valid also for the picrotoxin-sensitive type of post synaptic inhibition

Fig 7 illustrates a popliteal motoneurone of 63 mV monosynaptic spike height which was impaled in a spinalized and strychninized (0 08 mg/kg) cat In *A* the neurone was fired repetitively by injected current and brief manual stretches of the muscle tibialis anterior inhibited the discharge In *B* a high d c -gain shows the hyperpolarizing

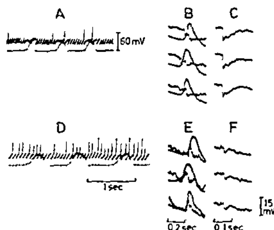


Fig 7 Strychninized (0 08 mg/kg) and spinalized cat Popliteal motoneurone of 63 mV spike height *A* Neurone fired by injected depolarizing current and brief manual stretches of the tibialis anterior muscle inhibited discharge *B* High d c gain revealed 6 0 mV hyperpolarizing shift of average membrane potential during manual stretch *C* Single electrical shock to common peroneal nerve caused 10 5 mV inhibitory post synaptic potential lasting around 100 msec Between *C* and *D* picrotoxin was administered i v (0 6 mg/kg) and recording procedure repeated *D* Manual stretches of tibialis anterior could not inhibit the discharge induced by injected current 4 min after the picrotoxin administration *E* The former hyperpolarizing shift of the membrane potential had been replaced by depolarizing response of shorter latency *F* The long lasting IPSP following stimulation of the common peroneal nerve had largely been removed by picrotoxin

shift of the membrane potential of 6 0 mV which occurred during stretch of the muscle A single shock to the common peroneal nerve caused an inhibitory post synaptic potential (*C*) which had a duration of 100 msec and an amplitude of 10 5 mV The rising phase of this IPSP was slow and lasted 8 msec In both *B* and *C* it was possible to reverse the direction of the inhibitory potential by passing hyperpolarizing current into the motoneurone (not shown in the figure) thereby excluding disfacilitation as the possible cause of the inhibitory events Picrotoxin was then administered i v in a dose of 0.6 mg/kg and after 4 min *D* was recorded Stretches of tibialis anterior could not inhibit the discharge induced by injected current In *E* a depolarizing shift of the membrane potential appeared during the rising phase of the muscle stretch Presumably this excitatory response had earlier been suppressed by the inhibitory effects which had now been removed by picrotoxin

There is also the possibility that picrotoxin had removed presynaptic inhibition so as to release excitatory afferents the effects of which then might obscure the remaining post synaptic inhibition However this does not seem likely when considering record *F* showing that the IPSP from stimulation of the common peroneal nerve likewise had been largely removed by the drug (compare with *C* of the same Fig) If presynaptic inhibition on primary afferents was activated by the peripheral stimulus in C and F its long latency (Eccles Eccles and Magni 1961) would not allow it to influence the induced afferent input There is therefore no reason to doubt that the strychnine resistant post synaptic inhibition in *A* and *B* also was removed by picrotoxin

In Fig 8 is illustrated another experiment from a spinalized and strychninized (0 08 mg/kg) cat It is a popliteal motoneurone of 72 mV spike height which was impaled with a potassium chloride micro-electrode In *A* inhibitory post synaptic potentials were evoked by applying single electrical shocks to the hamstring nerve These IPSPs had an amplitude of 4 5 mV and a duration of around 80 msec The slow rising phase of the IPSPs lasted 13 msec Chloride ions were then injected into the moto-

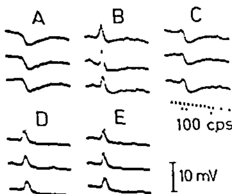


Fig 8 Strychninized (0 08 mg/kg) and spinalized cat Popliteal motoneurone of 72 mV spike height (KCl micro-electrode) *A* Single electrical shocks to the hamstring nerve evoked inhibitory post-synaptic po-

had been replaced by an earlier excitatory potential C. Two min after the chloride injection the IPSP returned Picrotoxin was

No difference is seen in *E* when comparing with *D*, however, showing that this strychnine resistant IPSP was completely removed by picrotoxin

neurone for 60 sec by passing a hyperpolarizing current of 35 nA through the micro electrode Immediately (2—4 sec) after the cessation of this injection *B* was recorded, showing that now but little remained of the inhibitory post-synaptic potential Instead an excitatory response had appeared which had 7 msec shorter latency than the IPSP, and which previously had been suppressed Two min after the injection of chloride ions (*C*) the IPSP had largely returned but the excitatory response was still present as a little hump preceding the inhibitory potential Picrotoxin was then administered i v in a dose of 1 2 mg/kg, and after 6 min *D* was recorded It is seen that the inhibitory post synaptic potential had disappeared and the excitatory potential reappeared

There is the possibility that the IPSP had not actually been removed by the drug but rather obscured by simulataneous excitatory activity at the post-synaptic membrane This proposition could be analyzed by increasing the intracellular concentration of chloride ions so as to convert the summated excitatory and inhibitory activity into a depolarizing response Chloride ions were therefore again injected into the motoneurone by passing hyperpolarizing current of 50 nA through the micro-electrode for 70 sec Comparing *D* with *E*, which was recorded immediately after the cessation of the chloride injection no obvious difference between the two records can bee seen, indicating that this strychnine-resistant IPSP had been removed by picrotoxin The resting membrane potential had the same value in all the records

Discussion

The results from the present study demonstrate the existence of a type of post synaptic inhibition in cat spinal motoneurones which contrary to current views (Curtis 1963 Eccles 1964 1965) is depressed or abolished by picrotoxin In no case was a post synaptic inhibition found to be resistant to both strychnine and picrotoxin In strychninized cats the remaining post synaptic inhibitions were always abolished by picrotoxin It cannot be generally concluded from this relationship that all peripherally activated picrotoxin-resistant inhibitions are sensitive to strychnine, and vice versa but since the present results can be fully accounted for by only two types of post-synaptic inhibi-

tion it is hardly necessary at this stage to speculate on the possibility of other types peripherally activated post-synaptic inhibitory mechanisms

It has previously been claimed that picrotoxin does not influence post synaptic inhibition in the spinal cord (Eccles 1964) but this statement derives from experiments in which the effects of picrotoxin apparently were studied on post synaptic inhibitions of short latencies (Eccles *et al* 1963) or else were found to be strychnine sensitive (Desmedt and Monaco 1960) In the present investigation the picrotoxin sensitive post synaptic inhibitions were found to arrive along polysynaptic pathways and to exceed in latency the monosynaptic spike by at least 3 msec

The long latency of this inhibition may suggest that the afferent information ascends to supraspinal levels and returns along previously known strychnine resistant pathways (Bremer 1925, Terzuolo 1954 Llinas 1964) However since strychnine resistant post synaptic inhibition was easily obtained in spinalized cats this explanation seems superfluous It appears on the contrary, that picrotoxin sensitive post synaptic inhibition occurs at the spinal level Furthermore picrotoxin sensitive post synaptic inhibition may explain the convulsive activity caused by picrotoxin, which in the past could not be fully accounted for by the depression of presynaptic inhibition (Eccles 1964 1965)

The picrotoxin sensitive inhibitory post synaptic potentials produced by electrical stimulation of peripheral nerves were generally large and had a slow rising phase (usually around 10 msec) They also had a duration of 80—100 msec, only occasionally shorter see e g Fig 5) At the moment it is not known whether this long duration is caused by prolonged transmitter or by temporal spread of the synaptic activity Strychnine resistant IPSPs of similar configuration have been described in supra spinal areas e g Andersen *et al* 1963) but sensitivity to picrotoxin has not been found (Crawford *et al* 1963)

To distinguish between true post synaptic inhibition and disfacilitation as the cause of reduced excitability of the motoneurone the presence of hyperpolarizing synaptic activation noise is of importance as it clearly reveals inhibitory activity at the post synaptic membrane itself (Granit Kellert and Williams 1964 *a b*) Its absence may however suggest disfacilitation but it might also indicate post synaptic inhibitory activity at a site which is remote with respect to the micro-electrode tip and hence produces activation noise not detectable by the latter In order to avoid this diagnostic difficulty another criterion for post synaptic inhibition was adopted, namely its sensitivity to an increased intracellular concentration of chloride ions (Coombs *et al* 1955 Eccles *et al* 1964 In all cases tried it was possible to abolish or reduce picrotoxin sensitive inhibitory post synaptic by injecting chloride ions into the motoneurone There was also complete or partial recovery of the IPSP some time after completion of the chloride injection showing that this type of inhibitory mechanism has a chloride dependent equilibrium potential In a number of cases intracellular chloride injections were applied after the administration of picrotoxin in order to reveal remaining inhibitory activity see Fig 8)

In no case however was it possible to reverse a picrotoxin sensitive IPSP into a depolarizing response even after using protracted chloride injections and strong currents This is in contrast to the reversal of the very chloride susceptible direct inhibitions Coombs *et al* 1955 Eccles *et al* 1964) An explanation of this difference may be that the synapses responsible for the picrotoxin sensitive post synaptic inhibitions are largely situated out on the dendrites whereas the induced ionic changes are mainly restricted to the soma where the electrode is supposed to be impaled However since

it was possible to record picrotoxin sensitive IPSPs all of the synapses of this type can not be remote (Frank 1959, Granit *et al* 1964*b*) Llinas and Terzuolo (1964), studying the descending inhibition from the reticular formation on flexor motoneurones obtained similar results after intracellular chloride injections and explained them on basis of dendritic localization of the inhibition

two different types of synapses one of which produces terminal depolarization the other neurone hyperpolarization Picrotoxin may also act earlier in the interneuronal chain but further studies are needed to clarify this point

Some of the characteristics of picrotoxin sensitive post synaptic inhibition found in the present study and in a previous paper (Kellerth and Szumski 1966) may be summarized as follows the inhibition is a) resistant to strychnine or even b) potentiated by strychnine c) removable by picrotoxin (picrotoxin-sensitive), d) possesses an important pathway in flexor muscle afferents acting on extensor motoneurones and e) is mediated by long polysynaptic pathways

Since all the properties listed above are also given as characteristic for presynaptic inhibition the relative importance of these two types of picrotoxin sensitive inhibitions creates an interesting problem for further investigation In the present study the post synaptic type was frequently found to be activated by muscle stretch Except for the study by Mendell and Wall (1964) on pressure-activated cutaneous afferents there is very little information on the role of presynaptic inhibition with natural stimulation (see *e g* the review by Bennet 1964) In their study on presynaptic inhibition Devanandan Eccles and Yokota (1964) used muscle stretches of 10 msec duration a type of stimulation that hardly would occur under natural conditions and which with regard to synchronization does not differ significantly from electrical stimulation of peripheral nerves

Another main problem concerns the different roles and the interplay of the two types of peripherally activated post synaptic inhibitions one of which is sensitive to strychnine the other to picrotoxin Since they are activated by different transmitter mechanisms it is not unlikely that different ionic mechanisms are involved at the post-synaptic membrane As shown by the present results and the study by Coombs *et al* (1955) however both types of inhibition are affected by an increased intracellular concentration of chloride ions

An even more complex situation arises when the descending inhibitions from higher centres are taken into consideration The study by Llinas (1964) who investigated the influence of reticular stimulation on spinal extensor motoneurones suggests the existence of a third type of post synaptic inhibition resistant to both strychnine and picrotoxin This type of inhibition was also found to be affected by intracellular injection of chloride ions

It is therefore concluded that much work remains to be done in order to elucidate the various types of post synaptic inhibition and the integration of the information which is conveyed to the post synaptic membrane of individual cells by different transmitter mechanisms

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References

ANDERSEN, P, J C ECCLES Y LØYNING and P E VOORHOEVE Strychnine resistant inhibition in the brain *Nature* (Lond) 1963 *200* 843—845

BENNETT, M V L, Nervous function at the cellular level *Ann Rev Physiol* 1964 *26* 289—340

BREMER, F, Recherches sur le mechanism de l action de la strychnine sur le système nerveux *Arch int Physiol* 1925 *25* 131—152

COOMBS, J S, J C ECCLES and P FATT, The specific ionic conductances and the ionic movements across the motoneuronal membrane that produce the inhibitory post-synaptic potential *J Physiol* (Lond) 1955 *130* 326—373

CRAWFORD J M D R CURTIS P E VOORHOEVE and V J WILSON, Strychnine and cortical inhibition *Nature* (Lond) 1963 *200* 845—846

CURTIS D R, Pharmacological investigations upon inhibition of spinal neurones *J Physiol* (Lond) 1959 *145* 175—192

CURTIS D R, The depression of spinal inhibition by electrophoretically administered strychnine *Int J Neuropharmacol* 1962 *1* 239—250

CURTIS D R The pharmacology of central and peripheral inhibition *Pharmacol Rev* 1963 *15* 333—364

DESMEDT J E and P MONACO Suppression par la strychnine de l'effet inhibiteur centrifuge excercé par la faisceau olivo-cochléaire *Arch int Pharmacodyn* 1960 *129* 244—248

DEVANANDAN M S R M ECCLES and T YOKOTA, Presynaptic inhibition induced by muscle stretch *Nature* (Lond) 1964 *204* 996—998

Ec... 1964

Ec...

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ECCLES J C R M ECCLES and F MAGNI, Central inhibitory action attributable to presynaptic depolarization produced by muscle afferent volleys *J Physiol* (Lond) 1961 *159* 147—166

ECCLES J C P FATT and K KOKETSU Cholinergic and inhibitory synapses in a pathway from motor axon collaterals to motoneurones *J Physiol* (Lond) 1954 *126* 524—562

ECCLES J C R F SCHMIDT and W D WILLIS Pharmacological studies on presynaptic inhibition *J Physiol* (Lond) 1963 *168* 500—530

FRANK K Basic mechanisms of synaptic transmission in the central nervous system *IRE Trans Med Electronics ME 6* 1959 85—88

GRANIT R J-O KELLERTH and T D WILLIAMS Intracellular aspects of stimulating motoneurones by muscle stretch *J Physiol* (Lond) 1964a *174* 435—452

GRANIT R J O KELLERTH and T D WILLIAMS "Adjacent" and remote post synaptic inhibition in motoneurones stimulated by muscle stretch *J Physiol* (Lond) 1964b *174* 453—472

KELLERTH J-O and A J SZUMSKI Two types of stretch activated post synaptic inhibitions in spinal motoneurones as differentiated by strychnine *Acta physiol scand* 1966 *66* 133—145

... pharmacological ... 37 ... ses- ...

28 413—422

MENDELL L M and P D WALL Presynaptic hyperpolarization A role for fine afferent fibres *J Physiol* (Lond) 1964 *172* 274—294

TERZUOLO C A Influences supraspinales sur le tétanos strychnine de la moelle épinière *Arch int Physiol* 1954 *62* 179—196

UNRATH K Der fermentative Abbau der Erregungssubstanz der sensiblen Nerven seine pH-Abhängigkeit und seine Hemmung durch zentral erregende Stoffe Naunyn—Schmiedeberg's *Arch exp Path Pharmak* 1953 *219* 148—155

Acta physiol. scand. 1966. 66. 157—163

From the Institute of Physiology, University of Lund, Sweden

A Comparison of the Biological Value of Various Types of Swedish Bread and the Effect of Added Lysine

By

STIG LARSSON

Received 20 May 1965

Abstract

LARSSON, S. *A comparison of the biological value of various types of swedish bread and the effect of added lysine.* Acta physiol. scand. 1966. *66.* 157—163. — Feeding experiments on rats given diets made from various types of bread widely used in Sweden are reported. It was found that the addition of L-lysine to all diets increases the activities of glutamic-oxalacetic transaminase (GOT) and ornithine-carbamyl transferase (OCT) of the liver. At the same time the total [illegible]

In previous publications the loss of added lysine and threonine during the baking of wheat bread and of soft bread with high content of reducing sugar was studied (Ericson, Larsson and Lid 1961 a, Ericson and Larsson 1962). It was found that the loss of lysine during baking of wheat bread was small (5—15 %), whereas the loss of threonine was considerably higher. During the baking of soft bread with high contents of reducing sugar (VR-bread) the loss of supplementary L-lysine HCl was high (approximately 30 %). It was also found that the protein value of VR-bread in itself was low, probably due to inactivation of the protein bound lysine caused by reactions with reducing sugar.

Increased growth does not necessarily mean increased protein deposition (Allison 1949). In recent years it has been found that a low protein value will not only give lower growth rate as compared with feeds of high protein value but also a change in carcass composition towards higher fat deposition (*cf.* Clausen 1963, Munck 1964 and Larsson, Nilsson and Olsson 1966).

Therefore it seemed of interest to study the effect of lysine supplementation to different types of Swedish bread.

Experimental

Analytical Procedures

slightly longer time

For further details concerning the microbiological amino acid determinations the reader is referred to Ericson *et al* (1961*a*)

The nitrogen contents of the diets was determined by the Kjeldahl method described by Perrin (1953)

(1957) The ornithine-carbamyl transferase (OCT) activity was measured according to Reichard (1957)

The rats were then frozen and homogenized in a Waring blender together with a known amount of water The homogenate was analyzed for percentage of dry matter and fat as described above The carcass protein was determined according to Lowry *et al* (1951)

The bread used as feed and the baking conditions

The wheat bread was pan baked from a dough having the same composition as previously described (Ericson *et al* 1961*a*) The VR bread had the same composition and was subjected to the same baking conditions as described previously by Ericson and Larsson (1962)

The crisp bread (knackebrod) was of commercial brand When purchased the water

water used for making the dough To 91 % the air dried and ground bread was added 3 % salt mixture (Hegsted *et al* 1941) 5 % soya bean oil 0 5 % cold liver oil and a vitamin mixture (Harper *et al* 1953) The diets were frozen until used

Experimental animals

Male rats of the Sprague Dawley strain were used At the start of the experiments they weighed approximately 50 g The animals were housed individually in cylindrical glass containers with wood shavings The temperature in the animal room was 25° C with a relative humidity of 50 % Before the experiments the animals were given the basal wheat bread diet for four days from the time of weaning The diets were given *ad libitum* Tap water was freely available to the animals

Results

Table I shows the composition of the various diets used in the experiment The nitrogen contents of the different diets is highest in school bread due to a larger amount of dried skim milk

It may be seen that the growth rates of the rats given the basal wheat bread crisp bread and school bread diets are essentially the same Basal VR bread or rye bread

TABLE I Type of bread diet, nitrogen contents of feed, supplementation of L-lysine HCl decrease of added amino acid during baking and the growth rate in g per day of rats fed the various diets 20 animals in each group were used Feeding period 35 days *Mean±SEM Differences calculated according to Student's t test*

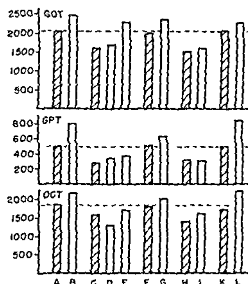
Diet	Nitrogen content %	Decrease of added L-lysine HCl during baking (determined microbiologically) %	Growth rate g/day
A Basal wheat bread	2 10		1 3±0 12
B A +0 40% L-lysine HCl added before baking	2 13	8	4 1±0 21[1]
C Basal VR bread	1 72		0 3±0 06
D C +0 40% L-lysine HCl added before baking	1 92	28	1 1±0 17[1]
E C +0 40% L-lysine HCl added after baking	1 91		1 3±0 14[1]
F Basal crisp bread	2 07		1 2±0 10
G F +0 40% L-lysine HCl added after baking	2 09		2 5±0 18[1]
H Basal rye bread	1 93		0 7±0 06
I H +0 40% L-lysine HCl added before baking	*1 93*	*31*	*1 0±0 06*
K Basal school bread	2 26		1 4±0 11
L K +0 15% L-lysine HCl added before baking	2 26	16	3 0±0 22[1]

[1] P< 0 001

TABLE II The effect of the different diets on weight water and fat contents of rat liver 20 rats in each group were used and the diets given for 35 days Means±SEM Differences calculated according to Student's t test

Diet	*Weight* g	*Water* %	*Fat %* on dry wt
A	4 6±0 19	72 1±0 32	17 7±0 45
B	8 2±0 18[1]	2 2±0 21	14 6±0 46[1]
C	2 6±0 07	72 2±0 41	24 0±0 42
D	4 0±0 14[1]	73 5±0 35	19 1±0 51[2]
E	4 3±0 72[2]	73 5±0 34	17 6±0 63[2]
F	4 5±0 13	74 8±0 27	17 4±0 71
G	*7 2±0 21[2]*	*74 1±0 34*	*12 9±0 53[3]*
H	3 8±0 10	74 7±0 26	18 3±0 43
I	4 6±0 11[3]	73 8±0 28	16 8±0 54
K	4 9±0 19	71 9±0 34	16 2±0 61
L	7 5±0 17[2]	70 2±0 27	16 1 0 47

[1] P<0 001 [2] P<0 01 [3] P<0 05



of ornithine-carbamyl transferase (OCT) o
rat liver The rats were given the diets fo
35 days Twenty animals in each grou
were used Enzyme activities expressed i
units per g wet weight

TABLE III Carcass contents of protein and fat of rats given the different diets for 35 day (20 rats in each group) Mean ± SEM Differences calculated according to Student t test

Diet	Protein % of body wt	Fat % of body wt	Protein/ Fat
A	17 8	14 2±0 71	1 25
B	19 1	8 3±0 81[1]	2 30
C	16 9	16 3±1 30	1 04
D	17 1	14 5±0 80	1 18
E	17 9	12 5±0 81[2]	1 43
F	18 0	14 8±1 01	1 22
G	19 1	10 3±0 68[2]	1 85
H	17 2	15 7±1 05	1 10
I	17 6	13 3±0 60	1 32
K	17 9	15 0±0 93	1 19
L	19 0	9 3±0 90[1]	2 04

[1] P 0 001 [2] P 0 01 [3] P 0 05

diets given to t result in significantly low tes The addition of L-lysine HCl to the ts in all cases improved t es read and school bread diets est growth rate respo plementation When com basal VR bread d n increased

growth rate 3—4 times However the basal value was exceedingly low — growth rate 0.3 g per day

Table II indicates that the livers from rats on the supplemented diets all increased in weight and that the fat content of the liver decreased on these diets Fig 1 gives the activites of GOT, GPT, and OCT of the livers from the rats subjected to the different diets The activities seem to increase in the livers from animals on the supplemented diets when calculated on equal liver weight basis When the values are calculated on total liver however, expressing the capacity of the liver the difference is still more accentuated

Table III indicates that lysine supplementation depresses the fat deposition of the body as a whole on account of increased protein deposition

Discussion

The data obtained in the present study give the biological values for different types of bread on the Swedish market The biological value or protein value may be calculated after the growth rates obtained after feeding different diets As pointed out by Allison (1949) however growth is not necessarily equivalent to a gain in body nitrogen (protein) When studying effects of different diets with regard to biological values of food one therefore desires to obtain values not only from growth rate curves but also from fat and protein deposition The importance of such measurements has previously been pointed out (Clausen 1963 Munck 1964 Larsson *et al* 1966)

contents of the liver The growth rate of rats given rye bread was also low In this connection it is of interest to note that VR bread and rye bread are the types mostly consumed in Sweden Further the high fat content of the livers of animals given the basal VR bread diet should be emphasized together with the low values of GOT GPT and OCT Decreased transaminase activities of liver have been observed in protein defficiency Srinivasan and Patwardhan 1955) In the present study the VR and rye bread diet could not give rise to a protein deficiency *per se* On the other hand due to a low food intake a relative deficiency could be possible Larsson *et al* (1965) found in pigs that lysine methionine supplementation to a high protein diet (grits supplemented with animal protein increased the GOT activities of liver and skeletal muscle The addition of 0.40 % L-lysine HCl to the VR bread before or after baking led to a drastic improvement in both growth and efficiency of food utilization Further the fat deposition decreased as compared with the basal VR diet This agrees with the previous results by Ericson and Larson (1962) The present experiment also shows *that the fat contents of the livers from rats given lysine supplementation to the VR bread* decreased from 24.0 % to 17.6 % and that the activities of GOT and OCT drastically increased At the same time as mentioned above a change in carcass composition in favour of protein deposition was noted GOT-activity of livers was found to increase in pigs more than 80 % when 0.15 % L-lysine HCl and 0.05 % DL-methionine was added to a commercial pig feed Larsson *et al* 1966 This finding indicates that not only protein deficiency as such but also the protein quality will cause adaptive changes in some transaminating systems The fat contents of the liver from animals given the

supplemented wheat bread was significantly lower compared with the controls (basal wheat bread) which agrees well with previous studies (Ericson, Larson and Rubarth 1962)

The present results have shown an increased fat deposition, relatively, on account of protein deposition on diets with low biological value The increased fat deposition gives a certain relation as to the etiology of certain types of obesity Too much emphasize on overweight has been brought into the discussion instead of taking into account the body composition The present results show that two diets with the same nitrogen and caloric contents can produce so marked difference in carcass composition that on a diet with low biological value the animals can be regarded as obese However there are experimental data indicating that not only the biological value of the diets are of importance for the etiology of obesity Thus, hereditary factors seem to be of importance with regard to carcass composition On one and the same diet, different strains of mice will vary with regard to carcass composition (Larsson 1966) In the strains studied so far, however, the protein value of the diet is of importance particularly in strains where the carcass composition of the animals "normally" has a tendency towards high fat deposition Cohn and Joseph (1960) found that the rate of ingestion of foodstuffs plays a significant role in the regulation of intermediary metabolism Further, "meal eating", e g feeding the animals for 1 or 2 hrs a day significantly increased fat deposition in the body In the present study all animals were fed *ad libitum*, and here the fat deposition was found to be related to the biological value of the protein These observations suggest multiple causes of obesity in animals with intact nervous regulation of food intake

In a previous study (Ericson and Larsson 1962) it was found that the loss of added lysine during the baking of soft bread with high contents of reducing sugar is comparatively high (25—30 %) This finding was verified by the present experiments As mentioned before, the loss of added lysine during the baking of regular wheat bread was only about 10 % The VR bread was again found to be an extremely poor protein source Available data from previous and present experiments seem to indicate that the loss of lysine during the baking of VR bread is not the only explanation for the poor biological value Earlier studies (*cf* Ericson *et al* 1961*a* and *b*) suggest that protein bound and free lysine can be made nutritionally unavailable by reactions between the ε amino group of lysine and carbohydrates or other compounds containing aldehyde groups

It is generally considered that rye and wheat with regard to biological value of protein are equal This might hold true for unprocessed products, but when rye is baked as soft bread this assumption is not correct The present study indicates that VR bread and rye bread both have an inferior biological value As discussed above this is partly *due to the loss of lysine during the baking procedure It has been found* however that malted rye has a deleterious effect on animals — possibly due to toxic products (Munck 1965 pers comm The process of baking is different for the various types of bread and most certainly influences the biological value Thus in Sweden the soft breads containing rye meal are usually baked at higher temperatures and for a longer time than for example wheat bread Also other procedures connected with the baking of VR bread and rye bread no doubt will facilitate enzymatic changes that would decrease the biological value as well as the appetite of the animals

Theoretically the biological value of school bread should be higher than for example wheat bread among other things due to higher contents of dried skim milk As already shown by Ericson *et al* 1961*b* the loss of biological value of dried skim milk during baking is remarkably high This might in part explain the absence of superior effect of

school bread with regard to growth rate compared to wheat bread Further, the carcass analysis revealed that the two diets were equal

At present there are evidence to state that not only quantitative data of the composition of diets with regard to total contents of protein or nitrogen are enough to determine the biological value of a feed or food Processing of diets has a profound effect (Reimus 1955) as has storing of food The present experiments seem to indicate that data from composition tables of diets give only rough estimates Thus, as has been pointed out above, the effect of heating the ingredients during the process causes reaction products between amino acids, reducing carbohydrates and free fatty acids which could not be metabolized by animals and man

References

[illegible]

ERICSON, L -E , S LARSSON and G LID, The loss of added lysine and threonine during the baking of wheat bread *Acta physiol scand* 1961*a* *53* 85—98

ERICSON L -E , S LARSSON and G LID A comparison of the efficiencies of free lysine and of roller dried skim milk, fish protein and soya bean protein for the supplementation of wheat bread *Acta physiol scand* 1961*b* *53* 366—375

ERICSON L E , S LARSSON and S RUBARTH, The effect of supplementation of wheat bread with amino acids and proteins on liver and muscle composition *Acta agricult scand* 1962 *12* 234—250

[illegible]

[illegible]

HEGSTED, D M , R C MILLS, C A ELVEHJEM and E B HART, Choline in the nutrition of chicks *J biol Chem* 1941 *138* 459—466

LARSSON, S Carcass composition, blood glucose and free fatty acids in various strains of mice *Acta physiol scand* 1966 In press

[illegible]

MUNCK, L The variation of nutritional value in barley II The CBA mouse as test organism for large-scale evaluations of nutritional characteristics in programs of plant breeding *Hereditas* (Lund) 1964 *52* 49—58

MUNCK, L., Pers comm 1965

PERRIN, C H Rapid modified procedure for determination of Kjeldahl nitrogen. *Analyt Chem* 1953 *25* 968—971

REICHARD H Determination of ornithine carbamyl transferase with microdiffusion technique *Scand J clin Lab Invest* 1957 *9* 311—312

REIMUS, L. Growth failure caused by mild heat treatment of the diet *Acta physiol scand* 1955 *35* 265—271

[illegible]

[illegible]

STEELE, B F H E SAUBERLICH M S REYNOLDS and C A BAUMAN Media for Leuconostoc mesenteroides P-60 and Leuconostoc citrovorum 8081 *J biol Chem* 1949 *177* 533—544

Acta physiol. scand. 1966, 66, 164—171

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Respiratory and Circulatory Effects of CO_2 Administered Gastroenterally

By

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Abstract

Landin A. M., O. Malmborg, O. Nisell and S. Zetterquist *Respiratory and circulatory effects of CO_2 administered gastroenterally.* Acta physiol. scand. 1966. 66. 164—171. — Pure CO_2 was injected through a tube into the stomach of 15 subjects for at least 15 min and with a rate of 50–300 ml per min. The CO_2 administration caused a proportional increase of the ventilation by 1.4–6.3 l/min. The simultaneous increase of CO_2 expiration (43–264 ml/min) was less than expected from the amount given, implying an accumulation in the body of 6–30 per cent of the CO_2 injected. The changes in arterial CO_2 tension were small, while blood sampled in 7 cases from the pulmonary artery showed more marked increases. There was also a significant rise of the oxygen uptake and of the heart rate, with a concomitant tendency to an increase of the cardiac output. The pulmonary vascular resistance decreased in 6 cases out of 8.

The increased quantity of CO_2 formed in the muscles during physical exercise is transported with the venous blood to the lungs. The possible significance of CO_2 for regulation of the circulation and ventilation during exercise might be studied by experimentally raising the CO_2 in venous blood. Such studies have earlier been made to a limited extent, the CO_2 having been administered in gaseous form intravenously (Eppinger *et al.* 1924, Scribner 1930, Coller and Paulon 1950), intraperitoneally and rectally (Loeper *et al.* 1933) or through the intravenous infusion of blood containing large quantities of CO_2 (Yamamoto and Edwards 1960, Cropp and Comroe 1961). It has been found that CO_2 can also be administered in considerable quantities through a tube to the stomach (Nisell 1957). The aim of the present investigation was to study by this technique the effect of gastroenterally given CO_2 on ventilation and central circulation in man, and thus to try to illustrate the ability of CO_2 to contribute to the functional adaptation to physical work. Preliminary results have been reported earlier (Landin, Nisell and Zetterquist 1961).

TABLE I Characteristics of subjects

Subjects	Sex	Age yrs	Height cm	Weight kg	Clinical diagnosis
1 TJ	♂	18	177	66	Healed duodenal ulcer
2 TS	♂	24	176	72	Normal
3 GS	♀	28	170	55	Colitis
4 MU	♀	26	162	54	Headache
5 KN	♂	18	174	59	Duodenal ulcer
6 PP	♀	23	150	48	Hypothyreoidism
7 PL	♂	28	171	69	Normal
8 HC	♂	17	166	52	Functional systolic murmur
9 AA	♀	21	168	66	Functional systolic murmur
10 LD	♂	34	180	85	Atrial septal defect
11 ML	♂	19	185	60	Pectus excavatum
12 KA	♂	20	177	65	Healed myocarditis
13 SÅ	♂	17	192	98	Functional systolic murmur
14 IM	♀	29	161	56	Functional systolic murmur
15 GP	♀	46	162	53	Mitral stenosis

Subjects and methods

The subjects were 13 patients from the medical department of Serafimerlasarettet and 2 healthy young men Some anthropological data and the clinical diagnoses are given in Table I The studies were performed in the morning with the subject fasting and recumbent In 8 cases they were made in connection with diagnostic heart catheterization

Fig 1

Expired air was collected for 5 or 10 min in airtight bags before, during and after the CO_2 administration (Table II The amount of air sampled was measured with a gas meter and the O_2 and CO_2 contents were analysed with a Haldane apparatus

During the air collection blood was withdrawn from a catheter percutaneously inserted into a brachial artery The CO_2 tension of the blood was obtained by measuring the pH by means of a glass electrode and a potentiometric pH meter directly and after equilibration of the blood at two known CO_2 pressures (Astrup 1956 The alveolar ventilation and physiological dead space were calculated see Comroe et al

Catheterisation of the pulmonary artery was performed in subjects no 8—15 The tip of a double-lumen catheter was wedged into a peripheral pulmonary vessel during an inspiration Pulmonary artery and capillary venous pressures were simultaneously recorded with electrical strain-gauge manometers Elema Sweden The difference between the means of these two pressures was taken as the pulmonary vascular pressure gradient The cardiac output was determined according to the Fick principle Pulmonary vascular resistance was estimated from the quotient of the pressure gradient and the cardiac output Heart rate was obtained from the electrocardiogram taken during the pressure recording

TABLE II. Data on CO_2 administration

Subject	Duration of adm. (min)	Amount CO_2 given (l (ATPD))	RQ at end of adm.	Estimate of stored CO_2 at end of adm. (l (STPD))	CO_2 balance after adm.[1] (ml STPD/min)	Times of measurements after start of adm.[2] (min)
1	60	12.18				60, 120
2	60	15.36	1.28	2.9	−5	60, 120
3	60	9.06	1.20	0.73	−8	60, 120
4	45	7.05	1.18	2.1	−36	15, 30, 45, 75, 105
5	40	9.20	1.36	2.2	+15	20, 40, 65, 95
6	90	9.60	1.13	2.1	−10	30, 60, 90, 120, 150
7	60	11.50	1.20	1.3	−52	20, 40, 60, 80, 100, 120
8	20	2.20	1.07	0.52	4	20, 43
9	17	2.81	1.11	0.30	17	17, 30
10	15	2.87	1.09	0.29		15, 30
11	20	4.66	1.26	0.61	27	20, 35
12	20	6.06				20, 46
13	22	6.60	1.46	0.42	10	22, 48
14	20	4.56	1.35	0.95	3	20, 50
15	18	1.35	0.90	0.29	11	18, 38
Mean	37.8	7.00	1.20	1.13	11.7	

Adm. = CO_2 administration.
[1] At first measurement after end of CO_2 administration.
[2] Measurements were completed at times noted.

Results

During the administration of CO_2 the subjects as a rule experienced no discomfor[t] except rumbling or gurgling in the stomach. A feeling of tension or mild pain in the abdomen sometimes occurred but quickly passed when the administration of gas was diminished. Eructation occurred seldom and no gas was given off from the anus.

During the CO_2 administration minute ventilation and CO_2 expiration increased with the administered quantity of CO_2 (Fig. 1 and 2). Oxygen consumption rose during the CO_2 administration in 10 of 13 cases (Table III). The mean increase was 23 ml STPD/min ($p < 0.01$). The magnitude of the ... did not appear to be correlated to the quantity of CO_2 administered per m... the CO_2 administration w... alveolar ventilation during of the physiological dead ... in all ca... ($p < 0.001$) and the increase CO_2/O_2 ratio at the e... 0.02) (Table III). The mally 1.46 (Table II). ... 70 and maxi... Fig. 3 ... in detail in ...

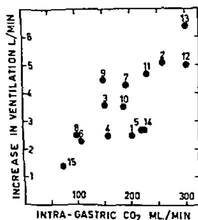


Fig 1.

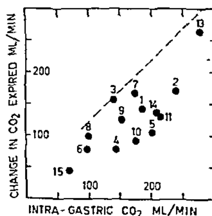


Fig 2

Fig 1 Increase of ventilation (litres BTPS/min) caused by gastroenteral CO_2 administration (ml ATPD/min) The increase is the difference between the ventilation during CO_2 administration and the mean value before and after administration The numbers beside the points relate to the respective subjects.

There was a certain increase of the heart rate, averaging 6 beats/min (Table IV), during the CO_2 administration ($p < 0.01$) This generally also implied an augmented cardiac output, but the difference was not significant Furthermore there was an increase of the pulmonary vascular pressure gradient ($p < 0.005$), while the corresponding vascular resistance decreased in 6 out of 8 cases The arterial CO_2 tension was mostly somewhat elevated during the CO_2 administration, but the difference from the value of the most basal control period (mean 1 2 mm Hg) was only of probable significance ($p < 0.05$) The effect on the CO_2 tension of pulmonary artery blood was more marked (mean 5 7 mm Hg) in the 7 cases in which it was determined ($p < 0.01$), as is shown in Fig 4

The respiratory and circulatory changes had already taken place in the earliest measurements which were made within 20 min after the start of administration of CO_2 but in the presentation of the results the mean values of the observations in each case have been used

Discussion

Large quantities of CO_2 — up to 250 ml min for 60 min — were administered gastroenterally without mentionable discomfort With no evidence of gas escaping through the orifices of the gut, this indicates a quick and complete absorption of the administered CO_2 from the stomach and intestine

TABLE III Some respiratory data

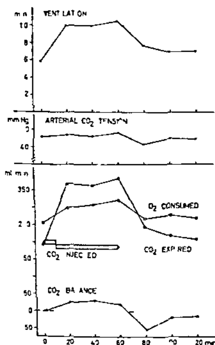
Subject	Control values					Change during CO_2 administration		
	$\dot{V}$ l/min	$\dot{V}_{CO_2}$ ml/min	$\dot{V}_{O_2}$ ml/min	PA_{CO_2} mm Hg	$\dot{V}_A$ l/min	$\dot{V}_{O_2}$ ml/min	$\dot{V}_A$ l/min	V_D ml
1	7.3	244		41.5	4.2		2.5	
2	5.4	194	243	41.0	4.1	39	4.2	20
3	5.9	195	244	41.0	4.2	54	2.9	90
4	6.0	165	208	36.8	3.9	13	2.5	30
5	7.9	264	271			−10		
6	4.6	133	185	36.0	3.2	8	1.2	10
7	5.8	183	255	46.0	3.4	45	3.3	70
8	7.6	180	210	36.2	4.3	50	2.0	5
9	6.1	153	232	34.2	3.9	22	3.8	20
10	11.0	187	244	28.6	5.8	23	2.9	50
11	5.2	141	240	40.1	3.1	−16	2.8	170
12	6.8	197	252	39.5	4.4		5.8	
13	8.0	241	310	35.0	6.0	38	6.7	−48
14	6.0	163	199	35.0	2.9	29	3.1	31
15	11.0	215	282	28.0	6.6	0	0.6	0
Mean	7.0	190	241	37.1	4.3	23	3.2	37

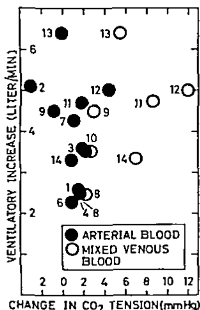
$\dot{V}$ = total ventilation (BTPS), $\dot{V}_{CO_2}$ = CO_2 expired (STPD), Pa_{CO_2} = arterial CO_2 tension, $\dot{V}_A$ = alveolar ventilation (BTPS), V_D = physiological dead space (BTPS)

TABLE IV Data on pulmonary circulation

Subject	Heart beats/min		Cardiac output l/min		Pressure gradient mm Hg		ΔR mm Hg / l/min	$P\bar{v}_{CO_2}$ mm Hg
	X	Δ	X	Δ	X	Δ		
8	86	10	7.7	1.8	9.3	0.7	−0.10	33.0
9	73	5	7.5	2.5	4.0	−0.2	0.29	36.2
10	81	6	13.6	3.0	11.0	2.0	−0.09	31.0
11	73	2	7.3	1.6	4.0	1.0	0.33	43.5
12	48	9	7.5		4.8	1.2		41.0
13	60	9	8.9	0.9	4.8	0.7	0.10	37.5
14	72	0	[illegible].9	0.5	4.3	1.0	0.12	37.0
15	100	8	4.4	0.6	28	2	0.25	32.4
Mean	74	6	7.9	1.1	8.8	1.1	0.05	37.2

X = mean of values before and after CO_2 administration
Δ = difference between value during CO_2 administration and X value
ΔR = Δ for pulmonary vascular resistance (can not be calculated from values in this table)
$P\bar{v}_{CO_2}$ = CO_2 tension of mixed venous blood before CO_2 administration except in subject 8 in whom it was obtained 23 minutes after end of CO_2 administration



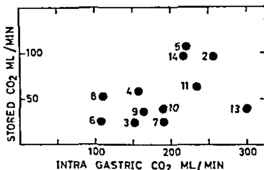


F g 3

F g 4

F g 3 Vent lat on (BTPS) arterial CO tens on O consumpt on (STPD) exp red CO_2 STPD) CO_2 adm n stered (ATPD and CO balance (STPD in subject 7

F g 4 Change of vent la on (l tres BTPS m n caused bv gastroenterally adm n stered CO_2 nd rel ed to s n ultaneous change of CO tens on n blood from the pulmonary and brach al arteries The values represent the d fference between the cond on during CO_2 adm n s rat on and the n ost basal value before or after adm n strat on



F g 5 Average quan v of re a ned CO (ml STPD m n n the bod n g s roen eral adm nis ra on of CO n rela on to the quan v adm nis- ered ml ATPD n n

The p lmonary excret on of CO_2 increased but not to an extent correspond ng to the amount of CO_2 gi en I i l hus a pos t e d fference v as found between the sum marized rate of CO_2 product on an l admin strat on and the rate of CO excret on in

dicating an accumulation of CO_2 in the body (Fig 5) The endogenous CO_2 production was calculated from the O_2 consumption determined during the CO_2 administration and the respiratory quotient of the control period From the duration of the CO_2 administration the total amount of CO_2 storage was also estimated (Table II) The quantity of CO_2 retained in the body amounted to 6—30 per cent of the amount given In pulmonary tissue the solubility of CO_2 has been shown to be about 0 8 ml/kg/mm Hg (DuBois *et al* 1953) If a similar value is presumed to be valid for abdominal organs and with the high gastroenteral CO_2 pressure used in the present investigation, it is reasonable to expect a considerable storage of CO_2 in the tissues surrounding the ventricle and intestine The protracted depletion of these stores is evidenced from the persistence of a high CO_2 excretion rate after the end of the CO_2 administration (Table II, Fig 3

The administration of CO_2 caused a significant increase of the ventilation Apparently the correlation was good between the ventilatory increase and the amount of CO_2 given (Fig 1) The simultaneous changes in arterial CO_2 tension were small and of the same order of relation to the increase of ventilation as those found by Alexander *et al* (1955) in subjects breathing air with 3 % CO_2 Corresponding results were also obtained by Beckman and Tornquist (1964) during intra peritoneal CO_2 administration The arterial CO_2 tension is furthermore known to be influenced very little by physical exercise (Holmgren and Linderholm 1958, Barr *et al* 1964, and others) Evidently exogenous CO_2 administration is as well compensated by optimal ventilation as the increased endogenous CO_2 formation during exercise, presumably through the action of CO_2 dependent mechanisms In an abstract based on a similar study Salzman and Sieker (1962) suggested the existence of chemoreceptors in the pulmonary circulation The present finding of apparent correlation between the changes in the CO_2 tension of mixed venous blood during gastrointestinal CO_2 administration and the simultaneous increase of ventilation (Fig 4) might be interpreted as support for such a hypothesis

The observed increase of oxygen uptake during the CO_2 administration is partly referable to the increased respiratory work The oxygen consumption of respiratory muscles has been estimated to be about 1 ml O_2/l of ventilation (Campbell *et al* 1959 Milic-Emili and Petit 1960) As the present mean increase of oxygen uptake was 10 ml O_2/l increase of ventilation, some additional explanation is needed It is possible that the basality of the subjects was changed by the procedure of gas administration

The CO_2 administration was accompanied by a small but significant increase of the heart rate compared to the control periods, and there was also a tendency to an augmentation of the cardiac output It can not be completely excluded, however, that this stimulation of the central circulation too was mainly due to an unspecific effect of the experimental procedure The pulmonary vascular pressure gradient rose slightly during the CO_2 administration while the calculated pulmonary vascular resistance decreased in six out of eight cases Such a lowering of the resistance is in conformity with the reaction of isolated cat lungs when the CO_2 tension of the perfusing blood is elevated (Nisell 1951

References

ALEXANDER J K J R WEST J A WOOD and D W RICHARDS Analysis of the respiratory response to carbon dioxide inhalation in varying clinical states of hypercapnia anoxia and acid base derangement *J clin Invest* 1955 *34* 511—532

ASTRUP P A simple electrometric technique for the determination of carbon dioxide tension in blood and plasma total content of carbon dioxide in plasma and bicarbonate content in

separated' plasma at a fixed carbon dioxide tension (40 mm Hg) *Scand J clin Lab Invest* 1956 *8* 33—43

Barr, P-O, M Beckman, H Bjurstedt, J Brismar, C. M Hesser and G Matell, Time courses of blood gas changes provoked by light and moderate exercise in man *Acta physiol scand* 1964 *60* 1—17

Beckman M and B Tornquist, Koldioxidresorption från bukhålan *Opuscula Medica* 1964 *9* 1—3

Campbell, E J M E K. Westlake and R M Cherniack, The oxygen consumption and efficiency of the respiratory muscles of young male subjects *Clin Sci* 1959 *18* 55—65

[illegible]

Eppinger, H, L von Papp and H Schwarz, *Über das Asthma cardiale* Berlin Julius Springer 1924 pp 213—216

[illegible]

l action respiratoire et circulatoire du gaz carbonique introduit par voie intraperitoneale et intrarectale *Bull Acad Med Paris* 1933 *109* 598—602

Milic-Emili, G and J M Petit Mechanical efficiency of breathing *J appl Physiol* 1960 *15* 359—362

Nisell, O, The influence of blood gases on the pulmonary vessels of the cat. *Acta physiol scand* 1951 *23* 85—90

Nisell, O, The effect of posture and intragastric gas administration on the oral glucose tolerance test *Acta med scand* 1957 *157* 445—449

[illegible]

Acta physiol. scand. 1966 66. 172—181

From the Institute of Clinical Biochemistry, Rikshospitalet University of Oslo Norway

The Organ and the Subcellular Distribution of Palmityl-CoA: Carnitine Palmityltransferase in Man

By

KAARE R NORUM

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Abstract

Norum K R *The organ and the subcellular distribution of palmityl-CoA carnitine palmityltransferase in man.* Acta physiol. scand 1966 *66* 172—181 — The organ and the subcellular distribution of palmityl-CoA carnitine palmityltransferase have been studied in man Biopsies were taken from patients of either sex operated in general anesthesia The activity of the enzyme was assayed by measuring the rate of incorporation of (^{14}C)-carnitine into palmitylcarnitine when the tissue preparation was incubated with palmitylcarnitine, (^{14}C)-carnitine and CoA. The enzyme was found to be present in all the organs and tissues tested except red blood cells and serum The greatest concentrations of the enzyme were found in the liver and the cortex of the kidney Medulla of kidney placenta heart and adipose tissue had intermediate levels Muscle uterus, brain lung leucocytes and blood platelets had relatively little enzyme activity In all the organs and tissues most of the enzyme activity was found in the mitochondrial fraction, but significant amounts were always present in the microsomal fraction and the particle-free supernatant. The amount of the enzyme found in each organ seems to correlate with the known rates of fatty acid oxidation in the respective organ.

Fritz 1955 discovered that carnitine stimulates the oxidation of fatty acids in tissue homogenates and in slices The same year Friedman and Fraenkel (1955) showed that carnitine is acetylated enzymically according to the equation

(1) Acetyl CoA + carnitine ⇌ acetylcarnitine + CoA. In 1962 Bremer found that the L-isomer of acetylcarnitine is efficiently metabolized by mitochondria from a number of rat organs Bremer 1962 a He proposed that carnitine acts as a carrier of activated acetyl groups through the mitochondrial membrane, and that the stimulatory effect of carnitine on fatty acid oxidation also could be explained by a similar mechanism i e long-chain acylcarnitines formed extramitochondrially may represent activated fatty acids which easily can permeate into the mitochondria where the oxidation of the acyl groups takes place This hypothesis has been supported by a number of observations The fatty acid moiety of long-chain acylcarnitines is efficiently metabolized by intact mitochondria from different organs (Bremer 1962 b, Fritz and Yue 1963

Carnitine stimulates the metabolism of palmityl CoA incubated with mitochondria from heart and liver (Fritz and Yue 1963, Bressler and Friedberg 1964) Furthermore, the enzyme which catalyzes the formation of long chain acylcarnitines in analogy with the equation

(2) Palmityl CoA + carnitine ⇄ palmitylcarnitine + CoA, *i e* palmityl CoA carnitine palmityltransferase (trivial name Carnitine palmityltransferase) has been found in both the particulate and soluble subfractions of cells from different organs in several species (Bremer 1963, Norum 1963 Fritz and Yue 1963, Norum 1964) The carnitine palmityltransferase therefore seems to play a key role in the stimulatory effect of carnitine on long-chain fatty acid metabolism, and a study of the organ and subcellular distribution of the enzyme seems warranted The present communication reports results obtained with human tissues The results are discussed in light of the fatty acid oxidation in these tissues

Materials and methods

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tillation spectrometer

Tissues The tissue and organ biopsies were taken from patients of either sex operated in general anesthesia The patients did not suffer from metabolic or endocrinological diseases The [illegible]

5 m[illegible]
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Studies were performed to ensure that the method was suitable also for the present problems Fig 1 and 2 show the results obtained with whole homogenate mitochondrial and microsomal fractions and particle free supernatant of human liver The curves show that linearity existed with incubation times and protein concentrations suitable for the present purpose Similar results were obtained in control experiments with human kidney cortex, placenta and adipose tissue

The enzymic exchange of the carnitine moiety of palmitylcarnitine with (^{14}C)-carnitine is dependent on a to and fro course of reaction (2) It is obvious that the assay method will give inaccurate values if there exist active enzyme system in the tissue preparations which efficiently compete with the carnitine palmityltransferase for the palmityl CoA formed during the reaction (2) Such competing enzyme system (*e g* palmityl CoA hydrolase and enzymes which catalyze the esterification of α glycerophosphate to triglycerides or to complex lipids might explain

TABLE I Incorporation of (^{14}C)-carnitine into palmitylcarnitine in different subcellular fractions from human liver and kidney cortex

Mitochondria microsomes and particle free supernatant were incubated for 10 min with palmitylcarnitine, (^{14}C)-carnitine and CoA, either separately or in combinations (see methods) Amounts of tissue preparation used Liver 0 15 mg of mitochondrial protein, 0 65 mg of microsomal protein, and 4 2 mg of particle free supernatant protein Kidney cortex 0 20 mg of mitochondrial protein, 0 40 mg of microsomal protein, and 1 1 mg of particle free supernatant protein

Cellular subfraction	Palmityl (^{14}C) carnitine formed (cpm) Liver	Kidney cortex
Mitochondria	1,160	1,300
Microsomes	1,280	760
Particle-free supernatant	2,760	480
Mitochondria + microsomes	2 460	2,200
Mitochondria + particle free supernatant	3,650	1 840
Microsomes + particle free supernatant	3 640	1,390

the findings that the microsomal fraction and the particle free supernatant have considerably less activity of carnitine palmityltransferase than the mitochondrial fraction (see Table II) If such competing enzymes should lower the enzymic exchange of the carnitine moiety of palmitylcarnitine with labeled carnitine one should expect that the formation of palmityl(^{14}C)-carnitine in an incubation system which contained both mitochondria and microsomes or particle free supernatant should be considerably less than the sum of palmityl(^{14}C)-carnitine formed in incubation systems containing mainly mitochondria, microsomes or particle free supernatant In the experiments shown in Table I the mitochondrial fractions the microsomal fractions and the particle free supernatants were incubated separately and in combinations The table reveals that the formation of the palmityl(^{14}C)-carnitine in the "combined incubations were nearly equal to the sum of labeled palmitylcarnitine formed in the "separate" incubations These results suggest that the relatively low carnitine palmityltransferase activity found in the microsomal fractions and the particlefree supernatants were not due to other enzymes competing with the carnitine palmityltransferase for the palmityl CoA formed in the enzyme assay reaction

This standard enzyme assay mixture was used in the present study 0 12 mM of CoA 5 mM of GSH 0 5 mM of DL-palmitylcarnitine 0 1 M of Tris/HCl buffer (pH 7 5) 0 3 mM of L-(*carboxy*-^{14}C -carnitine and varying amounts of enzyme protein in a total volume of 1 ml The incubation was performed at 30° for 5 or 10 min The activity of the carnitine palmityltransferase is expressed in cpm incorporated into palmitylcarnitine per min of incubation under standard conditions Specific activity is given as activity per mg of protein The protein was determined by the micro-Kjeldahl procedure by using copper and selenium as catalysts and by distilling the ammonia in the Parnass-Wagner all-glass distillation apparatus No corrections were done for none protein nitrogen in the tissues

Results

Table II shows the activity of carnitine palmityltransferase in human organs and tissues The *total activities* of the whole homogenates give information about the enzyme activity in 1 g wet weight of the organ or tissue The total activities of the subcellular

TABLE II The organ and the subcellular distribution of carnitine palmityltransferase in man

The activities are expressed in cpm incorporated into palmitylcarnitine per min when the organ or tissue preparations were incubated with palmitylcarnitine (^{14}C) carnitine and CoA. Experimental details are given in the text. The total activities in the whole homogenates are activities present in one gram wet weight of the tissue or organ. The total activities for the leucocytes and blood platelets are activities present in leucocytes and platelets from one ml of blood. The total activities in the cellular subfractions are enzyme activities present in the mitochondrial fraction the microsomal fraction or particle free supernatant derived from 1 g wet weight of the organs or tissues. The specific activities are activities per mg of protein

Organ	Age / sex			Whole homogenate		Mitochondrial fraction		Microsomal fraction		Particle free supernatant	
				TA[1]	SA[2]	TA[1]	SA[2]	TA[1]	SA[2]	TA[1]	SA[2]
Liver	AJ	64	♀	26 000	160	10 000	610	3 200	86	3 300	35
Liver	OO	48	♂	44 000	200	21 000	720	3 800	92	4 400	76
Liver	EE	47	♂	47 000	240	23 000	840	7 900	190	6 700	64
Kidney cortex	TS	18	♂	24 000	150	7 900	450	2 400	180	5 300	69
Kidney cortex	NM	58	♂	26 000	170	8 500	410	1 900	130	4 000	55
Kidney cortex	JB	19	♀	23 000	170	11 000	580	6 900	160	2 500	40
Kidney medulla	JB	19	♀	7 300	62	3 000	240	680	70	1 300	31
Placenta	SN	43	♀	4 600	50	2 400	350	420	60	430	11
Placenta	LL	21	♀	6 700	70	2 500	370	320	68	720	21
Placenta	RA	34	♀	4 600	42	2 400	390	460	67	440	14
Heart	AB	40	♀	3 400	19	—		—	—	570	7
Heart	EN	65	♂	5 500	34	2 400	130	610	100	450	11
Muscle	KB	40	♀	2 500	14	540	160	260	54	130	2
Muscle	LE	29	♂	1 700	11	520	150	280	37	81	2
Uterus	MB	42	♀	440	5	160	64	110	16	84	2
Brain	EM	57	♂	1 900	18	1 600	77	150	20	40	2
Lung	AJ	64	♀	1 700	13	770	83	180	21	340	5
Lung	NL	40	♀	1 300	15	550	49	140	22	270	5
Red blood cells	LR	28	♀	0	0		—			—	
Serum	LR	28	♀	0	0	—		—	—	—	—
Leucocytes	LR	28	♀	25	28	—		—	—		—
Leucocytes	KR	32	♂	31	34	—		—	—		
Platelets				30	38	—		—	—	—	
Platelets				22	27	—	—			—	—
Platelets				19	24	—	—			—	—
Adipose tissue[3]	GA	60	♀		15	—	—	—	—		4
Adipose tissue[3]	HS	56	♂		14	—	88	—	27		7
Adipose tissue[4]	TS	18	♂	—	18	—	37	—	28		6
Adipose tissue[4]	ET	28	♂	—	26		210	—	55	—	9
Adipose tissue[4]	KB	32	♂	—	22	—	140	—	29	—	7
Adipose tissue[4]	TS	18	♂		20	—	100	—	30	—	11

[1] Total activity [2] Specific activity [3] Subcutaneous [4] Retroperitoneal

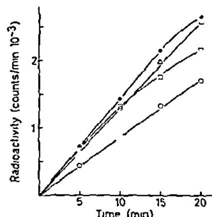


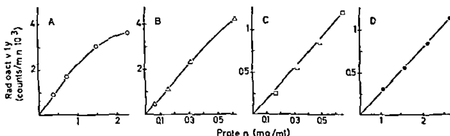
Fig 1 Incorporation of (^{14}C)-carnitine into palmitylcarnitine catalyzed by whole homogenate and subcellular fractions from human liver as a function of time Whole homogenate (○) 0[illegible] mg of protein, mitochondria (△) 0 15 mg of protein, microsomes (□) 0 65 mg of protein and particle free supernatant (●) 2 1 mg of protein were used Time varied as shown

compartments represents enzyme activities in the mitochondrial fraction in the microsomal fraction and in the particle free supernatant derived from 1 g wet weight of the organ or tissue Each horisontal row of figures represents values obtained from one biopsy For most of the organs and tissues values from 2 or 3 biopsies from different individuals are shown Although the number of individual biopsies investigated from each organ or tissue are quite small, the relatively little variance in the enzyme activity indicate that the present material gives a good indication about the amount of enzyme present in the different organs and tissues

In all the organs and tissues tested, the mitochondrial fractions contained most of the cellular carnitine palmityltransferase and the particle free supernatant usually contained more activity than the microsomal fraction In most of the organs and tissues tested the sum of the total activities found in the three subcellular compartments was considerably less than the total activity found in the whole homogenate We found that this loss of enzyme activity was mainly due to sedimentation of unbroken cells and mitochondria in the first centrifugation at 500 × g for 5 min Some activity was also lost in the washings of the mitochondrial fraction However, the values for the total activities present in the mitochondrial fraction and in the microsomal fraction and in the particle free supernatant give some indication of the relative distribution of the enzyme in the three subcellular compartments

Table II also shows the *specific activities* in the whole homogenates and the subcellular compartments from the organs and tissues In all the organs and tissues the mitochondrial fraction had the highest specific activity In most of the organs and tissues the specific activity of the microsomal fraction was about 1/4th of that found in the mitochondrial fraction No such pattern was found with respect to the specific activity of the particle free supernatant

Table II reveals that liver was the organ which contained the highest amount of carnitine palmityltransferase per g wet weight The liver biopsies were taken from patients with diseases of the stomach One of the patiens (A J) had considerably less total enzyme activity that the other patients The specific activities in the tissue preparations however, did not differ so much from the values of the other two patients A possible explanation of these findings could be an local edema in the tissue formed during the removal of the biopsy from A J



F g 2 Incorporat on of (¹C)-carnitine into palmitylcarnitine catalyzed by whole homogenate (A) mitochondria (B) m crosomes (C) and particle free supernatant (D) from human liver as a function of added protein Incubation t me 10 m n protein concentration varied as shown

The kidney biopsies were taken from tissue resected because of congenital kidney and kidney pelvis malformations Microscopial examinations were performed to exclude pathological material The cortex of kidney was found to have almost as much carnitine palmityltransferase as the liver The medulla of kidney had considerably less activity than the cortex It may be recalled that extensive oxidation of palmitate takes place in the kidney cortex whereas the oxidation of long chain fatty acids in the medulla is slight (Lee Vance and Cahill 1962)

The placenta specimens were taken from normal deliveries This organ have a high metabolic activity (Villee 1962) and we found that it contained considerable amounts of carnitine palmityltransferase

Among the muscular tissues the heart had the highest level of carnitine palmityl transferase activity The heart biopsies were taken from auricular tissue removed during commisurotomia on patients with mitral stenosis and the tissue most likely was not representative for the whole heart muscle The values obtained can therefore be considered minimum values The sceletal muscle biopsies were taken from the inter costal muscles Smooth muscle was taken from a pregnant uterus during a Caesarean section performed because of pelvic malformation

The brain specimen consisted of normal temporal gray matter removed during an internal decompressive craniotomy performed on a patient suffering from malignant glioma With the method used for the isolation of the brain mitochondria this fraction would be contaminated with broken nerve fibers (Stahl *et al* 1963) The specific activity found for the brain mitochondria therefore most likely is too low

The activity of carnitine palmityltransferase was also determined in the various components of blood No enzyme activity could be demonstrated in the red cells or in serum White blood cells and blood platelets however had specific activities of the enzyme of the same order of magnitude as found for heart muscle and brain No at tempts were done to isolate cellular subfractions from leucocytes and platelets by syste mically differential centrifugation In a few experiments however they were disinte grated by repeated freezing and thawing in 10 per cent sucrose — 0 01 M Tris buffer, and then centrifuged for 1 hr at 150 000 × *g* Nearly all the enzyme activity was found in the sediment

The carnitine palmityltransferase was also found to be present in adipose tissue It was difficult to test the enzyme activity in the whole homogenate of adipose tissue since the

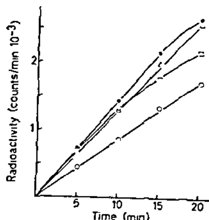


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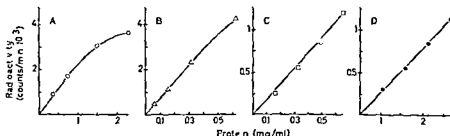


Fig 2 Incorporation of (¹⁴C)-carnitine into palmitylcarnitine catalyzed by whole homogenate (A) mitochondria (B) microsomes (C) and particle free supernatant (D) from human liver as a function of added protein Incubation time 10 min protein concentration varied as shown

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fat must be removed in order to get reliable assay conditions. This was done by removing the top fat layer after centrifugation of the homogenate for 5 min at 500 × *g*. The yield of protein in the water phase varied so much that no reliable data on the total activities could be given. In Table II, therefore, only specific activities are given. The fat free adipose tissue homogenate was fractionated by differential centrifugation in order to separate the mitochondrial and the microsomal fraction from the particle free supernatant. The same conditions as used for the other tissues and organs were employed. The results obtained suggest that the specific activities of the fat free whole homogenates and of the mitochondrial fractions from retroperitoneal adipose tissue were higher than the specific activities in the same fractions derived from subcutaneous adipose tissue. This may be in agreement with the higher metabolic activity found in the omental fat tissue in comparison with that found in the subcutaneous adipose tissue (Hamosh *et al.* 1963).

Discussion

There exist at least two different enzymes which catalyze the reaction

(3) Acyl-CoA + carnitine ⇄ acylcarnitine + CoA, one enzyme catalyzing the transfer of short chain fatty acids and one catalyzing the transfer of long chain fatty acids (Fritz, Schultz and Srere 1963, Norum 1963, Bremer 1963, Norum 1964). The mitochondrial carnitine acetyltransferase catalyzes the formation of acetylcarnitine from carnitine and acetyl CoA formed in the mitochondria. Several authors (Bremer 1962 a, Norum and Bremer 1963, Fritz *et al.* 1963, Bressler and Katz 1965) have proposed that acetylcarnitine may represent "active acetate" delivered from the mitochondria to the extramitochondrial compartments of the cell, where most of the synthesis of fatty acids and cholesterol takes place. Other authors, however, claim that the mitochondrial carnitine acetyl transfer system plays a minor role in the delivering of acetate to the extramitochondrial fatty acid synthesis (Lowenstein 1965, Srere 1965). Recent results may suggest that the carnitine acetyl transfer system is concerned with the formation of acetylcholine, as acetylcarnitine can act as acetyldonor for acetylcholine (Thomitzek und Strach 1964), and as the carnitine acetyltransferase is found in highest concentration in conductile tissues (Marquis and Fritz 1965).

As mentioned in the introduction to this communication, the carnitine palmityltransferase seems to play a key role in the stimulatory effect of carnitine on long-chain fatty acid oxidation. We found it therefore of interest to study the organ distribution of the enzyme and to correlate the enzyme activity in the organs with the rates of fatty acid oxidation in these organs. We have in the present investigation found that the enzyme is present in all the organs and tissues tested except red blood cells and serum. The concentration of the enzyme per g wet weight varies to a considerable extent in the different tissues. Liver and cortex of the kidney have the highest concentration of the enzyme. There is relatively little activity in lung, brain and muscle, while heart and placenta show intermediate values.

Human liver and heart oxidize *in vivo* about 0.1 μmole long chain fatty acid per min per g wet weight (Fritz 1961). It is known that about 80 per cent of the renal oxygen consumption is used in the oxidation of fatty acids (Lee *et al.* 1962). On that basis it can be calculated that 1 g of kidney also oxidizes about 0.1 μmole fatty acid per min. The oxidation of fatty acids in sceletal muscle *in vivo* is about 0.002 μmoles per min per g muscle (Friedberg and Estes 1962). These data correlate well with the high carnitine palmityl

transferase activities found in liver and kidney and the low activity found in muscle The reason why such a correlation is not so good in the case of heart may be that the auricular tissue tested is not representative for the whole heart with respect to the concentration of the enzyme Thus in the rat, the activity of carnitine palmityltransferase per g wet weight in heart and liver is of the same order of magnitude, and equals that found in human liver (Norum, unpublished results) It may therefore be suggested that there is a positive correlation between the amount of carnitine palmityltransferase present in an organ and the rate of fatty acid oxidation in that organ To the best of our knowledge there exist no data in the literature concerning the quantitative long chain fatty acid oxidation in human brain, adipose tissue, lung, placenta uterus, leucocytes and blood platelets The oxidation of long-chain fatty acids, however, have been shown to take place in some of these organs and tissues (Beattie and Basford 1965, Vignais Gallagher and Zabin 1958, Benjamin *et al* 1961, Elsbach 1963), and the concentration of carnitine palmityltransferase found in these organs and tissues may probably be correlated with their capacity to oxidize fatty acids

Our data on the specific activities of the carnitine palmityltransferase in the whole homogenates correlates well with the specific activities of the enzyme involved in fatty acid oxidation Thus, Wieland, Reinwein and Lynen (1956) found that liver, kidney and heart have the highest specific activity of 3 ketoacyl CoA thiolase (EC 2 3 1 16) and of 3 hydroxyacyl CoA dehydrogenase (EC 1 1 1 35) Lung, muscle and brain have low activities, while adipose tissue has an intermediate specific activity

Table II shows that, in all the organs and tissues tested, the mitochondrial fraction contains most of the cellular enzyme activity, and this cellular subfraction invariably has higher specific activity of the carnitine palmityltransferase than the other compartments of the cell However, both the microsomal fractions and the particle free supernatants contain significant amounts of enzyme activity We cannot exclude the possibility that the carnitine palmityltransferase activity found in the microsomal fractions was due to contamination with disrupted mitochondria Similarly, the particle free supernatants might be contaminated with soluble protein from disrupted mitochondria However, the activity of carnitine palmityltransferase found in the particle-free supernatant is most probably not derived from soluble mitochondrial protein as the mitochondrial enzyme activity is firmly bound to the mitochondrial membranes (Norum 1964) On the basis of the data in Table II and the above considerations it seems warranted to conclude that carnitine palmityltransferase in all the tissues and organs is localized mainly in the mitochondrial fraction but most likely not exclusively so

That carnitine may act as a "carrier" of activated long chain acyl groups through the mitochondrial membrane seems well established (Bremer 1962 b 1963, Fritz and Yue 1963, Bressler and Friedberg 1964, Bode und Klingenberg 1965) If this mechanism should be of physiological importance in the oxidation of long-chain fatty acids then long chain acylcarnitines must be formed outside or at the external surface of the membrane of the mitochondria and after they have penetrated into the mitochondria the reverse process *i e* a formation of acyl CoA s from acylcarnitines and mitochondrial CoA must take place Thus it may be suggested that the function of *mitochondrial* carnitine palmityltransferase is to catalyze the formation of acyl CoA's whereas the task of the *extramitochondrially* enzyme is to catalyse the formation of long-chain acylcarnitines In agreement with this view it has been found that isolated liver microsomes can synthesize palmitylcarnitine from palmitate and carnitine in the presence of CoA and ATP (Bremer 1963) However we have in the present investigation found that the cellular

carnitine palmityltransferase mainly is localized in the mitochondrial fraction and that relatively little enzyme is present extramitochondrially This skewed distribution could imply that the reversible acylation of carnitine catalyzed by the mitochondrial enzyme is a double sided membrane process, *i e* the mitochondrial enzyme is capable of reacting with its substrates on both sides of the membrane Thus, the enzyme might catalyze the formation of acylcarnitines from acyl CoA's and carnitine on the outside of the membrane, while on the inside the reverse reaction is taking place, giving acyl CoA's from acylcarnitines and mitochondrial CoA This hypothesis is in agreement with observations that the carnitine palmityltransferase is firmly bound to the mitochondrial membranes from disrupted mitochondria of calf liver (Norum 1964) and of human, rat, guinea-pig and pig livers (Norum, unpublished results) The observations that carnitine stimulates the oxidation of palmitate and of palmityl CoA in isolated mitochondria preparations (Fritz and Yue 1963, Bressler and Friedberg 1964 Beattie and Basford 1965) are also in agreement with this hypothesis

Although further experiments are required to elucidate the mechanism of the action of carnitine in the transfer of activated long chain groups into the mitochondria, the results obtained in the present investigation suggest that the carnitine palmityl transferase is of great physiological importance in the oxidation of long chain fatty acids

The author is indepted to Dr med J Bremer for helpful discussions Dr A I Sanderud at
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References

BEATTIE, D D and R E BASFORD Brain mitochondria III Fatty acid oxidation by bovine brain mitochondria *J Neurochem* 1965 *12* 103—111
BENJAMIN W A GELLHORN M WAGNER and H KUNDEL Effect of aging on lipid composition and metabolism in the adipose tissues of the rat *Amer J Physiol* 1961 *201* 540—546
BODE C und M KLINGENBERG Die Veratmung von Fettsauren in isolierten Mitochondrien *Biochem Z* 1965 *341* 271—299
BREMER J Carnitine in intermediary metabolism Reversible acetylation of carnitine by mitochondria *J biol Chem* 1962 a *237* 2228—2231
BREMER J Carnitine in intermediary metabolism The metabolism of fatty acid esters of carnitine by mitochondria *J biol Chem* 1962 b *237* 3628—3632
BREMER J Carnitine precursors in the rat *Biochim biophys Acta* (Amst) 1962 c *57* 327—335
BREMER J Carnitine in intermediary metabolism The biosynthesis of palmitylcarnitine by cell subfractions *J biol Chem* 1963 *238* 2774—2779
BRESSLER R and S J FRIEDBERG The effect of carnitine on the rate of palmitate incorporation into mitochondrial phospholipids *J biol Chem* 1964 *239* 1364—1368
BRESSLER R and R I KATZ The effect of carnitine on the rate of incorporation of precursors
leucocytes
tty acids by
carnitine Arch Biochem
by liver slices and

FRITZ I B Factors influencing the rates of long-chain fatty acid oxidation and synthesis in mammalian systems *Physiol Rev* 1961 *41* 52—129

FRITZ I B S K SCHULTZ and P A SRERE Properties of partially purified carnitine acetyl transferase *J biol Chem* 1963 *238* 2509—2517

FRITZ I B and K T N YUE Long-chain carnitine acyltransferase and the role of acylcarnitine derivatives in the catalytic increase of fatty acid oxidation induced by carnitine *J Lipid Res* 1963 *4* 279—288

Mass USA 1965 27—34

NORUM K R The existence of short and long-chain acyl CoA carnitine acyltransferases in heart mitochondria *Acta chem scand* 1963 *17* 1487—1488

NORUM K R Palmityl CoA carnitine palmityltransferase Purification from calf liver mitochondria and some properties of the enzyme *Biochim Biophys Acta* (Amst) 1964 *89* 95—108

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From the Department of Physiology, University of Goteborg, Sweden

The Functional Role of Some Autonomic and Behavioral Patterns Evoked from the Lateral Hypothalamus of the Cat

By

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Abstract

Folkow B and E H Rubinstein *The functional role of some autonomic and behavioral pattern evoked from the lateral hypothalamus of the cat* Acta physiol scand 1966 *66* 182—188 — The

dependent on the thalamic alarm are conditioned feeding motor activity

In a previous publication (Folkow and Rubinstein 1965) the effects of topical stimulation in the hypothalamic "feeding center" of the cat largely corresponding to nucleus hypothalamicus lateralis were described It was found that in awake, food-satiated cats such stimulation induced a fully developed feeding response in a limited number of cases being then associated with characteristic circulatory and gastrointestinal changes However the most frequently obtained response upon stimulation of electrodes placed in this brain area consisted of a nonaffective exploratory response without any food consumption in direct connection with the hypothalamic stimulation It is possible that *these two types of responses differ quantitatively rather than qualitatively*, the exploratory reaction being perhaps derived from an extended, overlapping hypothalamic area and representing a less intense or "fragmentary" excitation of the feeding

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center *per se* If so, one would expect that other excitatory, or inhibitory, influences on this hypothalamic center may to some extent determine whether a topical stimulation will produce a fully developed feeding response or only an exploratory response It was, indeed, occasionally observed how from one and the same electrode position stimulation parameters, which usually induced only an explorative response, could sometimes induce a feeding response

In the present study an attempt was therefore made to analyse at least some of the mechanisms involved in determining the animal's response to a topical stimulation of the hypothalamic feeding center

Material and methods

drawbacks For instance they reveal poorly any relaxation beyond the 'resting level and may

too much interference with the animal For such reasons the open tip catheters were considered

food

Two types of behavioral responses to the hypothalamic stimulations could be distinguished a) *Exploratory responses* where the animal displayed a nonaffective searching activity walking around sniffing the floor and the food tray

b) *Alimentary responses* where the animal during the stimulation period scratched and licked the tray as if actually trying to get at food that was not present True consumption during the hypothalamic stimulation was seen in case the animal had left some of the fish portion that was presented at each 3 min interval

Two cats were used for studying the effect of topical stimulation of the feeding center on a conditioned response for getting food which by training had reached a level well above 90 per cent correct responses These animals were conditioned by presenting a buzzer sound for 15 sec a food reward a piece of fish — was introduced into the cage 10 sec after the start of the

topical brain stimulation (100 cps 1 msec, 2—3 5 volts) started simultaneously with the buzzer sound but lasted only 10 sec These stimulation periods were introduced only in 6 or 7 of the daily trials and were distributed at random The effects of stimulation of two electrode contacts eliciting feeding or alarm responses respectively (see Folkow and Rubinstein 1965) were compared by measuring their influence on the reaction time and on the transit time

After completion of the studies in the awake cats they were used for experiments under

Results

The ten cats selected for the present experiments showed, when stimulated at electrod tips placed at the Horsley-Clarke coordinates H -O to H -6, A 10—11, Lat 2 5—3 5 two distinctly different behavioral patterns From more dorsal sections alimentar responses were induced and, ventrally to these, alarm responses (see Folkow and Rubin stein 1965) The effects of such stimulations could therefore be compared as to thei influence on the parameters studied

A The influence of the level of satiation on hypothalamically evoked alimentary responses

It was occasionally observed in the present animals that electrical stimulation of the hypothalamic feeding center did not regularly induced an alimentary response in the same, satiated cat Further, animals usually exhibiting only an exploratory response upon stimulation sometimes exhibited a fully developed alimentary response As the electrode position and the stimulation parameters were the same the possibility was explored whether the level of "hunger", considered to modify the excitability of the feeding center, could affect the animal's response to repeated, identical stimulations, while the fasting condition was gradually transformed to food satiation For this purpose three cats were utilized in repeated tests as described in Methods, and the means of the results are illustrated diagramatically in Fig 1 The figure shows that the level of spontaneous exploratory activity was relatively high in the fasting animals The first stimulation test performed during this fairly intense spontaneous exploration, induced clearcut alimentary responses (scratching, licking the food tray) in only about 20 per cent of the trials, while the exploratory activity was clearly enhanced in 60 per cent of the trials When the presentation of food was started the spontaneous exploratory activity was greatly increased After that food had been presented three times and readily consumed a second stimulation test was performed A further, clear accentuation of the exploratory activity could be noted in only 10 per cent of the trials, presumably because it was already nearly maximal Clear alimentary responses were seen in only 30 per cent of the trials — The presentation of small pieces of food was continued and after the sixth meal the spontaneous, exploratory activity had again decreased

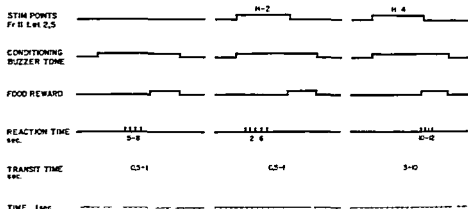


Fig 2 Diagram representing the effect of selective hypothalamic stimulation on a conditioned response for a food reward in the cat *Left column* After reaching 90 % correct responses the animals learned to react within 5—8 sec to the presentation of a buzzer sound lasting 15 sec This sound signalled the food reinforcement that was presented after 10 sec and withdrawn if not consumed with the cessation of the conditioning tone 5 sec later *Middle column* Topical stimulation of the "feeding center" (H-2) simultaneously with the presentation of the conditioning buzzer tone significantly shortened the reaction time (to 2—6 sec) without modifying the transit time. *Right column* Weak topical stimulation of the "alarm area" (H-4) considerably delayed the reaction time (to 10—12 sec) and retarded the transit time (to 3—10 sec), the animal showing a hesitant approach to the food tray

hyperventilation was observed Fig 2 illustrates the average findings in 5 sessions on each of the two cats

When no hypothalamic stimulation was performed the reaction time ranged from 5 to 8 sec (mean ± S E = 6 2 ± 0.28) and the transit time ranged from 0 5 to 1 sec When the *feeding center electrode was stimulated (H -2 in the figure)* a moderate but significant (p 0 01) decrease in reaction time was obtained the reaction time now ranging from 2 to 6 sec (mean ± S E = 4 7 ± 0 22), while the transit time was not significantly affected When the alarm area electrode (H -4 in the figure) was stimulated the situation was drastically different A highly significant (p = 0 001) prolongation occurred in the reaction time, which now ranged between 10 and 12 sec (mean ± S E = 11 3 ± 0 37 The transit time was also markedly increased, to 3 to 10 sec especially if a weak stimulation of the alarm area was continued, the animal interrupted its walk, looked around and hesitated

C. *Changes in gastrointestinal motility in the awake cat as a response to hypothalamic stimuli* —The gastric pressure changes were often misleading because of artifacts, like catheter displacement, obstruction possibly also because of an engagement of the vagal inhibitory fibres to the stomach (Jansson and Martinson 1965), in which case the catheter method will not reveal the events The recordings of the duodenal pressure changes were, however more regular and easier to analyse The effect on duodenal motility of hypothalamic stimulation was to some extent dependent on the type of prevailing spontaneous activity of which two main types could be distinguished 1) A slow rhythm (1—3 min) of relatively high pressure rises (20—40 cm H_2O), labelled "slow high", and 2) a faster rhythm (5—10 min) of low pressure rises (5—15 cm H_2O), labelled

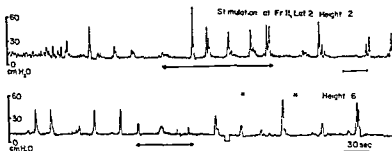


Fig 3 Cat 2 9 kg Recordings of duodenal press
cannula *1st row* A background activity of the "
"slow high type by topical electrical stimulation of
2nd row A background activity of the "slow high
topical stimulation of the "alarm area (Fr II La

fast low If the former type of spontaneous activity prevailed stimulation of feeding area electrodes tended to enhance further the rhythmic pressure rises If the spontaneous activity was mainly of the fast low type stimulation of the feeding area electrodes usually resulted in superimposition of relatively high pressure rises with a tendency towards a change in the pattern of activity to the slow high type this change often persisted for some time after the stimulation was turned off (top record of Fig 3, compare also the duodenal and jejunal responses recorded with the same technique in man before and after a meal Kewenter and Kock 1960) When such stimulation was started the cats reacted by approaching the food tray and usually but not always they consumed the available food

When on the other hand the animals were lightly stimulated via the alarm area electrodes so as to produce only increased alertness and slight mydriasis but no obvious increase in somatomotor activity there was a clear supression of prevailing slow high waves while fast low waves were less obviously affected (lower record of Fig 3

Discussion

The present experiments were designed to explore some functional characteristics of the behavioral patterns evoked by dorsolateral and ventrolateral hypothalamic stimulation in different test situations The results show that the extent to which dorsolateral hypothalamic feeding area stimulation induced alimentary responses depended on the previous feeding history of the animal Clearcut alimentary responses occurred most frequently at the beginning of satiation Further stimulation of the feeding area elicited a significant facilitation of an instrumental conditioned feeding response and an increase in intestinal motility earlier observed in anesthetised cats Folkow and Rubinstein 1965 seemed to be elicited by such stimulation in the awake animal as well

In marked contrast to these effects even weak stimulation of the ventrolateral hypothalamic alarm area greatly delayed and disrupted an instrumental conditioned feeding response and promptly suppressed the prevailing intestinal motility

These data support the assumption that each of the evoked behavioral patterns corresponds to an excitation of quite different integrative influences The "feeding pattern", evoked by dorsolateral hypothalamic stimulation, seems to facilitate the searching for, and the approach to food, whether these activities consist of an unconditioned appetitive behavior (see also Folkow and Rubinstein 1965) or a conditioned instrumental response Whether these effects of the stimulation may be called "motivational" and thus correspond to those described by Morgane (1961) as arising from the feeding center in the rat (a mid lateral "motivational" and a far lateral "feeding or metabolic") will require further experimental work with the possible role in mind that differences in species and methodology may play When the "alarm pattern" was evoked by topical hypothalamic stimulation it produced an inhibitory influence on the conditioned feeding responses, so that the unconditioned attack or escape responses completely dominated the behavior These results are in agreement with the findings of Knott, Ingram and Corell (1956) and Nielson, Doty and Rutledge (1958) in cats trained to press a lever for a food reward

The autonomic activation induced by stimulation through the feeding center electrodes illustrated in these experiments on awake animals by the enhanced duodenal motility, is in agreement with the earlier reported findings in anesthetized animals (Folkow and Rubinstein 1965) It is possible that the evoked autonomic response forms part of the so-called cephalic phase of gastrointestinal activation and that the stimulated hypothalamic zone therefore may be involved in the normal integration of the autonomic "anticipatory" adjustments of the gastrointestinal tract The increased intestinal motility induced by this stimulation, was usually associated with alimentary behavioral responses Whether these autonomically mediated gastrointestinal changes may facilitate the animal's orientation towards food and actual consumption by way of secondary afferent impulses from the gastrointestinal tract is not known so far If an exploratory activity is initiated by a feeding center stimulation, it seems reasonable to assume that the animal will be directed towards an actual food intake mainly because of the influence of signals coming from exteroceptors (smell taste sight) but signals from interoceptors such as gastrointestinal mechanoreceptors chemoceptors glucoreceptors etc might contribute significantly

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References

FOLKOW B and E H RUBINSTEIN Behavioral and autonomic patterns evoked by stimulation [illegible] 202—209

JA[illegible] relax[illegible]

[illegible] 0 119

KE[illegible]
430—438

KNOTT J R W R INGRAM and R CORELL Diencephalic influences on performance EEG clin Neurophysiol 1956 8 524

MORGANE P J Alternations of feeding and drinking behavior in rats with lesions in globi pallidi Amer J Physiol 1961 201 420

NIELSON H R W DOTY and L T RUTLEDGE Motivational and perceptual aspects of subcortical stimulation in cats Amer J Physiol 1958 194 427

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Thymic and Thoracic Duct Contributions to Blood Lymphocytes in Normal and Thyroxin-Treated Guinea-Pigs

By

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Abstract

in the thoracic-duct lymph

Exogenous thyroxin induces changes in the cell composition of the thymus, suggesting a disappearance of lymphocytes, possibly by emigration (Ernstrom 1963) However, it has never been conclusively demonstrated that thymic lymphocytes ever leave the thymus (Gowans 1964, Metcalf 1964) Thus, it is possible that all lymphocytes formed in this organ may remain there, die, undergo cytolysis and perhaps release nucleic acid or some other non-cellular factors into the circulation As it is generally claimed that the thymus contains few lymph vessels, the most probable main route for migration of lymphocytes from the thymus is the blood vessels The object of the present investigation — which is a continuation of a preliminary report (Ernström, Gyllensten and Larsson 1965) — was to establish whether such migration of lymphocytes from the thymus does, in fact, occur The lymphocyte content of afferent and efferent thymic blood has been determined Furthermore, the composition of the lymphocyte population of thymic-vein blood has been compared to that of thoracic-duct lymph

Exogenous thyroxin induces pronounced changes in the lymphocyte population of the blood, increasing the number of large lymphocytes characterized by a high mitochondrial content (Ernstrom and Larsson 1961, 1965) Even in thymectomized animals this thyroxin induced change occurs (although somewhat less pronounced), indicating the existence of an extrathymic source of these large lymphocytes in thyroxin treated guinea pigs The origin of these cells has been further investigated in the present paper by a comparison between the lymphocyte populations of the thoracic duct lymph and the thymic vein blood in normal and thyroxin treated guinea pigs

Material and methods

Totally 105 young male guinea pigs weighing 200—280 g were used Some were normal … Thyroxin … ligated at … ely) and …

After taking samples of venous blood from the thymus the right carotid artery was incised near the origin of the right thymic artery and a sample of carotid artery blood was taken f r a smear and for counting of white cells per mm³ of blood Immediately afterwards the artery was ligated

In some animals the right femoral vein was also incised and blood collected for a smear and white cell count

The confluence of the thoracic duct and the subclavian and jugular veins was exposed essentially according to the technique of Reinhardt and Yoffey (1957) although the sternum was not incised The thoracic duct was punctured a sample of lymph was taken in a pipette

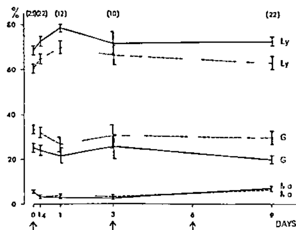
TABLE I Number of lymphocytes monocytes and granulocytes in thymic vein blood carot d … injection of thyroxin) The statistical comparison is based on differences in ind …

TV Thymic vein CA – Carotid artery FV Femoral vein Ly = Lymphocytes

TV			CA		
Ly	Mo	Gr	Ly	Mo	Gr
Normal animals					
3 495 ± 567 (10)	352 ± 53 (10)	1 716 ± 400 (10)	2 364 ± 220 (26)	244 ± 30 (26)	1 466 ± 231 (26)
Thyroxin treated animals					
3 919 ± 188 (12)	389 ± 77 (12)	1 227 ± 215 (12)	3 098 ± 515 (20)	345 ± 79 (20)	1 619 ± 347 (20)

[1] $p<0.05$ [2] $p<0.01$ denotes significant differences

Fig 1 Percentage of different white cells in thymic vein and carotid artery blood of normal controls and thyroxin treated guinea pigs Mean ± standard error Number of animals with n brackets The arrows indicate injection of thyroxin Ly = Lymphocytes Gr — Granulocytes Mo = Monocytes A higher incidence of lymphocytes is demonstrated in thymic vein blood than in carotid artery blood An increased percentage of lymphocytes is demonstrated 6 and 24 hours after a thyroxin injection Thymic vein blood ——— Carotid artery blood



per cell are denoted as a low medium and high mitochondrial content (MC) respectively
The blood smears were stained with Giemsa stain and the percentage of lymphocytes monocytes and granulocytes was calculated 200 white cells being differentially counted in each smear

artery and femoral vein blood of normal and thyroxin treated guinea pigs (9 days after initial dual animals Mean ± standard error Number of animals with n brackets

Mo Monocytes Gr = Granulocytes

FV			TV CA	TV FV	CA FV
Ly	Mo	Gr	Ly	Ly	Ly
2 376 ± 367 (19)	203 ± 29 (12)	1 384 ± 204 (12)	1 075 ± 293* (9)	851 ± 275* (8)	153 ± 189 (11)
3 148 ± 370 (18)	360 ± 77 (18)	1 395 ± 3 (18)	653 ± 473 (11)	809 ± 193* (11)	−46 ± 386 (17)

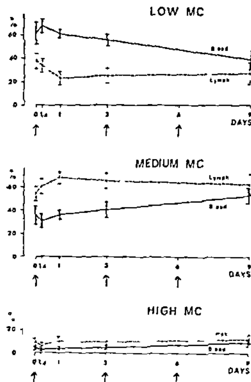


Fig 2 Percentage of lymphocytes with low, medium and high mitochondrial content (MC) in thoracic-duct lymph and thymic-vein blood of normal controls and thyroxin-treated guinea pigs. Mean ± standard deviation The arrows indicate injection of thyroxin. A higher incidence of lymphocytes with medium and high MC is demonstrated in the thoracic-duct lymph than in the blood In the thyroxin treated guinea pigs, an increased incidence of lymphocytes with medium and high MC is demonstrated in the thoracic-duct lymph, preceding a similar increase in the blood Thymic-vein blood ——— Thoracic-duct lymph - - - - -

For determinations of the absolute number of white cells, 25 mm³ of blood was diluted with 475 mm³ of Toisson's solution (containing methyl violet, for staining of white cells) The number of cells was counted in a Burker counting chamber, 288 squares (corresponding to 0 00625 mm³ each) counted for each sample

The results were analyzed statistically by Student's *t* test The comparisons between percentage and number of lymphocytes in thymic-vein and carotid artery blood were performed by statistical analysis of the differences in individual animals

Results

Blood lymphocytes

Normal animals The number of lymphocytes as a percentage of total white cells was higher in thymic-vein blood than in both carotid-artery blood ($n = 25, p < 0\,001$, see Fig 1) and femoral-vein blood ($n = 13, p < 0\,001$) Determinations of the absolute number of lymphocytes in both thymic-vein blood and carotid-artery blood proved that this percentage difference was due to a real thymic arterio-venous difference in lymphocyte content, and not to an elimination of other white cells (granulocytes, monocytes) from the blood during its passage through the thymus (Table 1)

Thyroxin treated animals As in the normal animals, the percentage of lymphocytes was higher in thymic-vein blood than in carotid-artery blood (Fig 1) In the animals investigated 6 hours, 1, 3 and 9 days after the initial injection of thyroxin, the following p values were obtained for the difference between arterial and venous blood $p < 0\,01$,

TABLE II Average mitochondrial content of lymphocytes in thymic vein blood and thoracic-duct lymph of normal and thyroxin treated guinea pigs 6 hours 1, 3 and 9 days after start of thyroxin treatment. Mean ± standard error Number of animals within brackets

Average mitochondrial content of lymphocytes				
	Thyroxin treated animals			
Controls	6 hrs	1 day	3 days	9 days
Thymic vein blood				
10 05±0 17 (33)	9 15±0 10[1] (24)	10 21±0 11 (12)	10 83±0 14[1] (12)	12 93±0 19[1] (24)
Thoracic duct lymph				
12 65±0 18 (29)	12 63±0 12 (14)	14 09±0 26[1] (11)	13 88±0 20[1] (12)	14 03±0 24[1] (24)

[1] $p<0.001$ denotes highly significant difference between thyroxin treated guinea pigs and controls

$p < 0.01$, $p < 0.1$ and $p < 0.01$, respectively In some of the animals investigated 9 days after the start of thyroxin treatment, the absolute number of lymphocytes was determined (Table I)

The number of lymphocytes as a percentage of total white cells increased after a single injection of thyro
at 24 hrs the increase
significant in carotid arte
again, and did not differ significantly from that in normal animals

The lymphocytes in thymic vein blood were classified by their mitochondrial content (MC) The mean MC of the lymphocytes was decreased 6 hrs after a single injection of thyroxin ($p < 0.001$) At 24 hrs, the mean MC of the lymphocytes in the thymic-vein blood had returned to the level in the controls and thus differed highly signifi-

1961, 1965) Thus, at 72 hrs the mean MC was increased highly significantly compared to the control level ($p < 0.001$) At 9 days the mean MC was further increased, highly significantly ($p < 0.001$) compared to the level at 3 days (Fig 2 and Table II)

Thoracic-duct lymphocytes

Normal animals The thoracic-duct lymphocytes contained a much greater percentage of lymphocytes with medium and high MC, and a correspondingly smaller percentage of lymphocytes with low MC, than did the blood lymphocytes (Fig 2) The mean

MC of the thoracic-duct lymphocytes thus exceeded that of the blood lymphocytes highly significantly ($p < 0.001$)

Thyroxin treated animals As in the normal controls, the mean MC of the thoracic-duct lymphocytes exceeded that of the blood lymphocytes highly significantly ($p < 0.001$) in all groups of thyroxin-treated animals (Table II)

In the animals treated with thyroxin, the percentage of lymphocytes with medium and high MC in the thoracic-duct lymph was increased above the normal level and the percentage of lymphocytes with low MC correspondingly decreased The mean MC was increased 24 hrs after a single injection of thyroxin ($p < 0.001$) Later, at 3 and 9 days from the start of thyroxin treatment, the mean MC showed no further significant change but remained on the high level (Fig 2 and Table II)

Discussion

The present investigation has revealed a thymic arterio-venous difference as regards the lymphocyte content per mm³ of blood Strong evidence is thus provided that lymphocytes leave the thymus through thymic veins It is apparent that the venous output of lymphocytes from the thymus exceeds a possible immigration of lymphocytes into it (*cf* Harris *et al* 1964)

The comparison between the lymphocyte populations of blood and thoracic-duct lymph shows that the lymph contains much more lymphocytes with medium and high mitochondrial content (MC) As the MC is correlated to the size of the lymphocytes (Wiseman 1931, Fichtelius and Larsson 1961), this also implies that the lymph contains relatively more large lymphocytes than the blood The different composition of lymph and blood as regards lymphocytes with different MC may be due to either or both of two events 1) *lymphocytes with higher MC disappear from the blood more rapidly than lymphocytes with lower MC* 2) *lymphocytes with low MC enter the blood directly (not through the thoracic duct) to a greater extent than lymphocytes with higher MC* Evidence of the former alternative has been given by Gowans (1964), who reported that labelled large thoracic-duct lymphocytes disappear rapidly from the blood and 'home' in the intestinal wall Evidence of the latter alternative is provided in the present paper and a following one

During treatment with thyroxin, the average MC of the blood lymphocytes is known to be greatly raised (Ernström and Larsson 1961, 1965) The present investigation has shown that this *increase in the blood is preceded by an increase in the thoracic-duct lymph* Thus, as soon as one day after injection of thyroxin, the mean MC of the thoracic-duct lymphocytes was increased, while that of the lymphocytes in the thymic vein blood was almost unchanged (as in arterial blood, see Ernström and Larsson 1961 The mean MC of the blood lymphocytes was increased 3 days from the start of treatment being still more pronounced after 9 days, whereas the average MC of the thoracic-duct lymphocytes was not further increased after day 1 *The findings strongly suggest that an increased number of lymphocytes with medium and high MC in the blood of thyroxin-treated animals enter the blood through the thoracic duct* In addition, we observed that an increased incidence of thoracic-duct lymphocytes with medium MC preceded a similar increase in thoracic-duct lymphocytes with high MC (Fig 2)

At 6 hrs after injection of thyroxin, the percentage of lymphocytes with low MC in thymic-vein blood was increased in comparison with untreated controls (Fig 2 This result, in combination with the finding of an increased percentage of blood lympho-

cytes out of total white cells (Fig 1) indicated the possibility of a thyroxin-stimulated venous output of small lymphocytes (characterized by a low MC) from the thymus This motivated a more detailed comparison between lymphocyte populations of afferent and efferent thymic blood in normal and thyroxin treated animals which will be reported in a following paper

References

ERNSTRÖM U Thyroxin induced changes in cell composition of lymph node tissue spleen and thymus *Acta path microbiol scand* 1963 *59* 145—155

ERNSTRÖM U and B LARSSON The influence of thyroxin on blood lymphocytes. *Acta path microbiol scand* 1961 *51* 17—23

ERNSTRÖM U and B LARSSON Influence of thyroxin on the mitochondrial contents of thymic and lymph node cells *Acta path microbiol scand* 1963 *59* 304—310

ERNSTRÖM U and B LARSSON Influence of the thymus on thyroxin induced changes in blood [illegible] 1965 [illegible]

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London 1964 255—273

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WISEMAN B K Criteria on the age of the lymphocytes in the peripheral blood *J exp Med* 1931 *54* 271—294

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Cholecystokinin and Pancreozymin, one single Hormone?

By

ERIK JORPES and VIKTOR MUTT

Received 12 June 1965

Abstract

Jorpes E. and V. Mutt *Cholecystokinin and pancreozymin one single hormone? Acta physiol scand* ... (PZ) are taken up ... ejunal ... e salt ... with 5 ... cellu ... oduct ... a Se- ... The ... on and ... n per ... h this rise in strength of the CCK activity as assayed in guinea pigs runs an equal rise in the PZ activity, as determined in cats at three different levels of strength namely at 0 3 220 and 3 000 (6,000) IDU/mg This observation strongly supports the assumption that both activities are exerted by one and the same substance It is also known that because of the methionine content of the purest polypeptide both are inactivated through the action of a dilute H_2O_2 solution, and both fully reactivated on subsequent reduction with cysteine hydrochloride

Of the many hormone like activities claimed to occur in extracts of the upper intestinal mucosa only the secretin cholecystokinin and pancreozymin activities have remained as established entities Secretin, the first endocrine substance, designated with the term hormone, was early shown to have a highly specific action stimulating the secretion from the pancreas of water and bicarbonate but not of enzymes In 1928 Ivy and Oldberg ascribed the gallbladder contracting activity of crude secretin preparations to a separate hormone named cholecystokinin (CCK) The correctness of this assumption has not since been questioned In addition Sandblom, Voegtlin and Ivy showed in 1935 that cholecystokinin preparations also exerted a dilating action on the sphincter of Oddi

As early as in the 1890 ies Pavlov had found that there is a dual mechanism regulating the pancreatic secretion, both a stimulus from the hydrochloric acid reaching the duodenum, eliciting a voluminous alkaline secretion, and a stimulus over the vagal nerve, causing secretion of a viscous juice rich in enzymes Since the latter effect could also be produced by injecting extracts of duodenal mucosa or preparations made from them Harper and Raper in 1943 suggested that there is a third hormone, analogous to secretin and cholecystokinin, which stimulates the secretion of enzymes from the pancreas together with secretin giving rise to a normal flow of pancreatic juice They named the hormone pancreozymin (PZ)

Harper and his coworkers elaborated a method of preparing PZ (Crick *et al* 1949) and studied the properties of the hormone (Harper and Mackay 1945/46, 1948, Harper 1946, 1959) They even applied it in clinical experiments The PZ preparations showed a marked CCK activity They could therefore be used in a series of investigations for the study of the functional state of the gallbladder and the patency of the common bile duct as seen in radiography (Duncan *et al* 1950, 1952, 1953, Howat 1952, Marks 1959, Harper 1960) Secretin and PZ were also used in combination as a test for the pancreatic function (Marks and Tompsett 1958, Burton *et al* 1960 a, b)

The purification of pancreozymin

All the three hormone activities mentioned are taken up on alginic acid from a dilute acetic acid extract of the heat coagulated duodeno-jejunal mucosa and, after elution from the alginic acid, precipitated together by sodium chloride (Jorpes and Mutt 1955 Mutt 1959 a, b) Secretin can be extracted from the dried salt cake with methanol, leaving a material with about 5 Ivy dog units of CCK per mg dry substance

By means of adsorption on a carboxymethyl cellulose column at pH 6 5 and fractionation with ethanol the activity was raised to 22—25 Ivy dog units of CCK per mg substance, as determined by Ivy and Janecek 1959

Professor Harper also kindly analyzed a sample of this type of preparation for its PZ activity It was found to be about 100 times higher than in his original PZ preparation, thus about 100—120 Crick, Harper and Raper units per mg

The preparations, exhibiting both CCK and PZ activities could now be injected into man although they still gave side reactions (Werner and Mutt 1954, Werner 1956, Jorpes and Mutt 1954, 1959)

Next followed chromatography on a TEAE cellulose column at pH 9 1 which led to a preparation with 220—250 Ivy dog units of CCK per mg (Jorpes and Mutt 1961) On this occasion we called attention to how closely the two hormone activities follow each other through all the steps of purification including chromatography on a weakly acidic and a strongly basic ion exchanger the strength of the PZ in the meantime having increased approximately 600 fold over that of the original standard preparation of Crick, Harper and Raper Side reactions with a sensation of heat in the face had now almost disappeared provided the preparation was injected slowly i v during the course of 1—2 min

At this stage of purity the PZ activity of the preparation was determined and the ratio CCK PZ compared with that in Harper and Raper's original pancreozymin preparation, given to us by Professor Harper in 1959 and in a recent sample of Pancreozymin Boots The two last ones had about the same CCK activity, 0 3 Ivy dog units per mg dry substance

TABLE I Pancreozymin activity of Pancreozymin Boots (A) charge No 23 with 0 3 Ivy dog units of cholecystokinin per mg as compared with that of a cholecystokinin preparation with 220 Ivy dog units of cholecystokinin per mg (B)

Sample	Dose Ivy dog units of cholecystokinin	Spectrophotometric reading (660 mμ)		
		Cat No 1	Cat No 2	Mean
—	(Secretin only)	0 055	0 058	
A	1 5	0 155	0 126	0 140
B	1 5	0 140	0 186	0 163
A	3 0	0 227	0 212	0 220
B	3 0	0 245	0 195	0 220
A	6 0	0 292	0 290	0 291
—	(Secretin only)	0 046	0 034	
B	6 0	0 310	0 282	0 296

Pancreozymin Boots charge no 23 containing per ampoule 91 units of PZ as defined by Crick, Harper and Raper in 1949, and 83 mg substance thus 1 1 PZ units per mg was thoroughly assayed for CCK activity by the Ljungberg procedure measuring the contraction of the gallbladder *in situ* in anesthetized guinea pigs against the CCK standard of the Pharmacopoea Nordica Ed Svecica Vol 4 Apotekarsocieteten, Stockholm (Ljungberg 1964) 0 2—0 8 ml of a solution with 0 25 Ivy dog units of CCK per ml was found to be a suitable dose for a single injection The Pancreozymin Boots was found to have a CCK activity of 0 3 Ivy dog units per mg substance The PZ activity stated by the label to be 91 Crick Harper and Raper units per ampoule thus 1 1 units per mg was then compared with that of a CCK preparation with 220 Ivy dog units of CCK per mg For that purpose 1 5 3 0 and 6 0 Ivy dog units of CCK of each of the two samples were injected in two anesthetized cats (Mutt and Soderberg 1959 under continuous secretin stimulation with 3 clinical units of secretin every 30 min Through a cannula in the pancreatic duct the juice was collected during 15 min after injection After another 15 min next injection was made The volume usually 1 5—2 ml was diluted to 25 ml with physiological saline solution and the protein content determined spectrophotometrically by means of the Lowry method Readings at 660 mμ The results are given in Table I

Evidently there is in the three different dilutions no considerable difference in the PZ activity if any between the two preparations and the ratio CCK PZ will be the same The PZ activity is proportional to the amount of CCK injected irrespective of the degree of purity of the preparation if 0 3 or 220 Ivy dog units of CCK per mg Thus on increasing the CCK activity of the preparation about 650 times there is an equal rise in the PZ activity

Gel filtration of the CCK preparation with 220 Ivy dog units per mg through a Sephadex G 50 column in 0 25 M phosphate buffer pH 8 0 (Fig 1), followed by chromatography on an Amberlite XE-64 column with a 0 05 M phosphate buffer pH 7 5 (Fig 2 resulted in a basic polypeptide fraction with a strength of 3 000—3 500

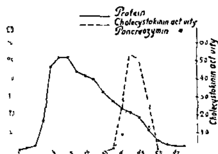


Fig 1

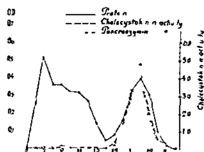


Fig 2

Fig 1 *Column* Sephadex G 50, fine, 1 5 × 90 cm *Buffer* Sodium orthophosphate, 0 25 M, pH 8 ± 0 1 *Chromatographed material* 100 mg PC—TEAE—C *Fraction volume* 5 ml *Flow rate* ca 8 min per fraction OD Optical density of fractions at 280 mμ Cholecystokinin activity of fractions, Ljungberg method (relative values) PZ Colour (Lowry) at 660 mμ of the pancreatic juice, diluted to 25 ml, of a cat after injection of equal aliquots of the fractions

Fig 2 *Column* Amberlite XE-64, 1 × 40 cm *Buffer* Sodium orthophosphate, 0 05 M, pH 7.5 ± 0 1 *Chromatographed material* 12 mg PC—SX *Fraction volume* 3 ml *Flow rate* ca 14 min per fraction

Ivy dog units of CCK per mg The polypeptide seemed to be only slightly contaminated (Jorpes, Mutt and Toczko 1964)

Now the PZ activity of a sample of the practically pure CCK with 3,000 Ivy dog units per mg was compared with that of the sample (no 363) with 220 Ivy dog units per mg, equal doses of the two as to CCK being given to a cat The results are given in Table II

Again there is on increasing the strength of CCK from 220 to 3,000 Ivy dog units per mg a proportional rise in the PZ activity Both entities have thus increased about equally, by a factor of 10 000 over the CCK and PZ activities of the Pancreozymin Boots and Harper's original PZ preparation

Table II Pancreozymin activity of two cholecystokinin preparations with 3,000 (A) and 220 (B) Ivy dog units of cholecystokinin per mg respectively

Sample	Dose Ivy dog units of cholecystokinin	Spectrophotometric reading (660 mμ) Cat No 1
—	(Secretin only)	0 054
A	6 0	0 770
B	6 0	0 740
A	12 0	0 910
B	12 0	0 770 (?)
A	24 0	1 220
—	(Secretin only)	0 125
B	24 0	1 100

TABLE III Pancreozymin activity of a cholecystokinin preparation (A) with 6,000 Ivy dog units of CCK per mg as compared with that of Pancreozymin Boots (B) with 0 3 CCK units per mg

Sample	Dose Ivy dog units of cholecystokinin	Spectrophotometric reading (660 mμ) Cat No 1
—	(Secretin only)	0 00
A	3 0	0 90
B	3 0	0 80
A	6 0	1 2
B	6 0	—
A	12	1 23
B	12	1 28
—	(Secretin only)	0 12
	(Secretin only)	0 07
B	3 0	0 86
A	3 0	0 67

Another sample of the Amberlite treated CCK which showed the strength of 6,000 Ivy dog units of CCK per mg, was carefully assayed for its CCK activity against the CCK preparation (No 363) with 220 Ivy dog units per mg and against Pancreozymin Boots Now the PZ activity of this sample was compared with that in Pancreozymin Boots (Lot no 23) (Table III)

In this experiment the dose 12 Ivy dog units of CCK was evidently too large As in the previous experiments, doubling the CCK dose produced a clear increase in the amount of protein eliminated with the pancreatic juice If the pancreozymin activity is related to the cholecystokinin dose administered, there is no distinguishable difference between the two preparations in spite of the 20,000 fold increase in strength both components in this case have undergone

The fact that the material in question has passed over an acidic ion exchanger CMC-cellulose a basic one, TEAE-cellulose, a Sephadex column and yet another acidic ion exchanger, Amberlite XE-64, resulting in a 10,000-(20,000-)fold increase in hormonal activity without any detectable change in the ratio CCK PZ speaks strongly in favor of the assumption that both activities are exerted by one and the same substance

Strong support for this assumption has been presented by Mutt 1964 who showed that the pure CCK which contains methionine in line with the pituitary adrenocorticotropic hormone the α and β-melanocyte stimulating hormones and the parathyroid hormone can be oxidized by hydrogen peroxide with complete loss of activity and reactivated to practically full strength with cysteine The pancreozymin activity simultaneously underwent the same changes Under similar conditions the pure secretin, which does not contain methionine, did not show any change in activity

Since cholecystokinin is known to act both on the gallbladder and on the sphincter of Oddi, an action on the embryologically closely related pancreas is not unlikely It

would also be in line with the finding of Gregory and Tracy (1964) that pure gastrin both G I and G II in dose of 2 μg/kg stimulates the pancreatic secretion in the dog a finding which was confirmed by Preshaw, Cooke and Grossman (1965) working with conscious dogs with a transplanted pouch of the pyloric gland area of the stomach The amount of CCK/PZ used in our cat experiments 0 1—0 4 μg/kg falls in about the same range of magnitude

The question that now remains to solve is whether the polypeptide carrying the CCK and PZ activities is a homogeneous chemical entity or not

References

Burton P D G Evans A A Harper H T Howat S Oleesky J E Scott and H Varley A test of pancreatic function in man based on the analysis of duodenal contents after administration of secretin and pancreozymin *Gut* 1960 *1* 111—124

Burton P E M Hammond A A Harper H T Howat, J E Scott and H Varley Serum amylase and serum lipase levels in man after administration of secretin and pancreozymin *Gut* 1960 *1* 125—139

Crick J A A Harper and H S Raper On the preparation of secretin and pancreozymin *J Physiol* (Lond) 1949 *110* 367—376

Duncan P R A A Harper H T Howat S Oleesky and H Varley The effects of [illegible] 63 P

[illegible] H Varley Tests of gall [illegible]

[illegible] Varley and J E Scott The use of the cholecystokinetic agent in preparations of pancreozymin to study gallbladder function in man *J Physiol* (Lond) 1953 *121* 19 P

Gregory R A and H J Tracy The constitution and properties of two gastrins extracted from hog antral mucosa *Gut* 1964 *5* 103—117

Harper A A and H S Raper Pancreozymin a stimulant of the secretion of pancreatic enzymes in extracts of the small intestine *J Physiol* (Lond) 1943 *102* 115—125

Harper A A and I F S Mackay A comparison of the effect on the pancreas of pancreozymin and of vagal nerve stimulation *J Physiol* (Lond) 1945/46 *104* 27 P

Harper A A The effect of extracts of gastric and intestinal mucosa on the secretion of HCl by the cat s stomach *J Physiol* (Lond) 1946 *105* 31 P

Harper A A and I F S Mackay The effects of pancreozymin and of vagal nerve stimulation upon the histological appearance of the pancreas *J Physiol* (Lond) 1948 *107* 89—96

Harper A A , Physiologic factors regulating pancreatic secretion *Gastroenterology* 1959 *36* 386—391

Harper A A The use of secretin pancreozymin and cholecystokinin in the diagnosis of pancreatic and biliary disease *Burma med J* 1960 *8* 127—134

Howat H T *Pancreatitis* In Modern Trends in Gastroenterology edited by F A Jones Butterworth & Co Ltd 1952 London

Ivy A C and E A Oldberg A hormone mechanism for gallbladder contraction and evacuation *Amer J Physiol* 1928 *86* 599—613

Ivy A C and H M Janecek Assay of Jorpes-Mutt secretin and cholecystokinin *Acta physiol scand* 1959 *45* 220—230

Jorpe[illegible]
zym[illegible]

Jorpe[illegible]

Jorpe[illegible] *36* 317—383

Jorpes J E and V Mutt The Gastrointestinal Hormones Secretin and Cholecystokinin Pancreozymin *Ann int Med* 1961 *55* 395—405

Jorpes E, V Mutt and K Toczko Further purification of cholecystokinin and pancreozymin *Acta chem scand* 1964 *18* 2408 2410

Ljungberg S Biologisk styrkebestämning av cholecystokinin *Svensk Farm Tidskr* 1964 *68* 351—354

MARKS I N and S L TOMPSETT, The diagnosis of pancreatic disease *Quart J Med* 1958 *27* 431—461

MUTT, V Behaviour of Secretin, Cholecystokinin and Pancreozymin to Oxidation with Hydrogen Peroxide *Acta chem scand* 1964 *18* 2185—2186

PRESHAW, R M A R COOKE, and M I GROSSMAN, Pancreatic secretion induced by stimulation of the pyloric gland area of the stomach *Science* 1965 *148* 1347—1348

SANDBLOM P, W dochoduodenal

WERNER, B and secretin and of

WERNER B, Inv 169—171

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Effects of Growth Hormone on Erythropoiesis in the Intact Rabbit and the Polycythemic Mouse

By

S. HALVORSEN

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Abstract

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influence through the pituitary gland cannot be stated with certainty ACTH or combined pituitary hormones injections did not, however, normalize the reticulocyte response to hypoxia in rabbits with lesions in the posterior hypothalamus (Halvorsen 1964) Although ACTH increased erythropoiesis in intact rabbits the response to

ACTH was different from the erythropoietic response to hypothalamic stimulation (Halvorsen 1963) It is known that growth hormone (GH) may also stimulate erythrocyte formation (Meineke and Crafts 1956), and it is the primary purpose of the present paper to report investigations on the effect of GH on erythropoiesis in the intact rabbit and to compare this effect with the effect of hypothalamic stimulation

The influence of GH on erythropoiesis has previously mainly been studied in hypophysectomized animals Most investigators have found that GH does not prevent the post hypophysectomy anemia although it causes a bone marrow hyperplasia and a peripheral reticulocytosis (van Dyke *et al* 1952 Gemzell and Sjostrand 1954 Fruhman *et al* 1954 Meineke and Crafts 1956) *In* intact mature rats Gaebler and Mathies (1951) found lower hemoglobin and hematocrit values following GH injections than in control animals The discrepancy between the bone marrow hyperplasia and peripheral reticulocytosis and the lack of rise in hemoglobin and hematocrit in hypophysectomized *animals given GH was explained by Fruhman et al (1954) as being due to hemodilution* Meineke and Crafts (1956) found however that the total red cell volume per 100 g b w was lower in GH injected hypophysectomized rats than in hypophysectomized controls The cause of this discrepancy is still unknown Studies on isolated perfused hindlegs with GH in the perfusate (Remmele 1963 Fisher *et al* 1964) provide evidence that GH has a direct effect on bone marrow

From the preliminary experiments with GH in the present series it was apparent that hemolysis or blood loss could theoretically explain the findings of bone marrow hyperplasia and peripheral reticulocytosis without increase but with a tendency to decrease in hemoglobin hematocrit and red cell volume These experiments raised the following problems Do GH injections cause peripheral red cell destruction resulting in a primary decrease in the red cell volume and a secondary bone marrow hyperplasia and peripheral reticulocytosis? Further studies failed to reveal significant blood loss and it seemed most reasonable to search for the effect of GH on erythropoiesis in the bone marrow itself As the polycythemic mouse does not have erythroid cells in proliferation or maturation it was decided to test the effect of GH in this animal. In order to study whether GH has an effect on the differentiation of stem cells into erythroid cells or on the later stages of proliferation or maturation in some experiments GH was given alone and in other experiments combined with erythropoietin

Material and methods

Male adult rabbits were used throughout the study The animals received daily injections of GH for 2 weeks four animals even for 4 weeks Human porcine[2] and ovine[3] GH preparations were used Rabbits no 1 and 2 received human GH in daily doses of about 0 5 mg kg b w Rabbits no 3 and 4 recieved porcine GH in doses of 1 I U/kg b w during the first week and
GH n doses f 8 I U kg
k The
stud
effect

[1] Human growth hormone kindly supplied by O Trygstad Pediatric Research Ins ute Rikshospitalet Oslo Method of Roos *et al* 1963
[2] Somactone kindly supplied by Ferring AB Malmo Sweden Method of C H Li (19
[3] Prolactin kindly supplied by Ferring AB Malmo Sweden. Method of C H Li (19

Before and after GH administration red cell mass (RCM) was determined with the Cr^{51} method (Halvorsen 1963) At the same time hemoglobin red cells and hematocrit were determined In 6 animals the determinations were performed after three days of GH treatment and in 4 animals after 4 weeks [illegible]

In 6 rabbits the red cell survival was determ[illegible]

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Results

[illegible] groups, but [illegible] to be of [illegible] from the because all parameters have been studied before and after a certain [illegible] importance, mainly rel[illegible] have been comp[illegible]

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[illegible] during the period of GH administration The hemoglobin and the number of erythrocytes [illegible] of the rabbits lost weight showed a moderate but statistically insignificant decrease

[1] FH-49 Friesecke-Hoepfner Erlangen — Bruck, Germany

TABLE I RCM RCM/kg body weight and hematocrit before and after a 2 week period of daily GH injections

GH treated group Two weeks

Rabbit no	RCM (ml)		RCM (ml)		Hematocrit	
	Before GH	After GH	Before GH	After GH	Before GH	After GH
1	44 9	46 8	20 2	18 9	42 0	41 3
2	47 2	47 4	18 4	17 5	41 3	43 0
3	43 9	46 3	18 8	19 3	43 0	44 3
4	58 0	52 4	20 6	18 0	38 7	40 6
5	45 1	55 6	17 5	23 7	35 0	39 6
6	50 3	52 9	19 7	16 5	45 2	40 5
7	49 7	48 6	16 6	21 1	41 7	38 9
8	50 5	48 6	17 8	21 6	37 3	36 7
9	51 2	55 6	20 8	20 6	40 0	39 3
10	52 2	54 4	21 4	19 2	40 0	37 0

TABLE II RCM RCM kg body weight and hematocrit before and after a 4 week period of GH injections

GH treated group Four weeks

Rabbit no	RCM (ml)		RCM/kg (ml)		Hematocrit	
	Before GH	After GH	Before GH	After GH	Before GH	After GH
5	45 1	51 2	17 5	16 5	35 0	37 0
6	50 3	62 2	19 7	19 0	45 2	36 5
7	59 7	45 2	16 6	15 6	41 7	41 6
8	50 5	45 7	17 8	14 8	37 3	36 0

Table II gives the values of RCM RCM/kg b w and hematocrit in 4 rabbits receiving GH for 4 weeks and Table IV compares the values with the results in the control series The RCM increased slightly and less than in the control rabbits The RCM/kg decreased and the hemoglobin hematocrit and the number of red cells also decreased slightly but insignificantly

Table III shows the changes in RCM hemoglobin and hematocrit after 3 days of GH treatment This time interval was studied because hemoglobin and hematocrit were lowest at that time in rabbits studied daily with these parameters *The RCM* decreased with a mean of 2 8 ml or 7 per cent of the initial value while 4 controls studied on consecutive days showed a mean increase in RCM of 0 4 ml or 0 5 per cent The actual loss of blood because of blood sampling could be calculated to be between

TABLE III RCM, hemoglobin and hematocrit before and after a three-day period of GH injections

GH treated group Three days

Rabbit no	RCM (ml)		Hemoglobin (gm)		Hematocrit	
	Before GH	After GH	Before GH	After GH	Before GH	After GH
9	51 2	47 3	13 2	11 3	40 0	35 0
10	52 2	51 3	13 5	12 0	45 2	36 5
15	40 2	49 3	14 4	12 0	43 2	38 6
16	30 7	26 9	12 6	10 5	39 3	33 8
17	37 2	30 1	13 9	11 3	39 2	34 0
18	45 0	41 9	15 8	11 2	44 7	38 2

TABLE IV Mean of the differences between the post and pre treatment valu s ± S E) The differences of the means between the GH treated and the control groups are tabulated in the third column

Parameter	2 weeks			4 weeks		
	GH (10)	Control (10)	GH − Control	GH (4)	Control (6)	GH − Control
RCM, ml	1 6 ± 1 3	1 8 ± 2 2	−0 2	2 2 ± 4 1	8 4 ± 4 1	6 2
RCM kg ml	0 5 ± 1 0	−0 4 ± 0 5	0 9	1 4 ± 0 5	−0 4 ± 1 1	1 0
Hematocrit	−0 3 ± 0 9	−0 9 ± 1 1	0 6	−2 0 ± 2 3	0 0 ± 2 5	−2 0
Hemoglo-bin g	−0 5 ± 0 4	−0 2 ± 0 3	0 3	−1 7 ± 0 7	0 0 ± 0 4	1 7
Erythro-cytes mill	−0 4 ± 0 2	0 2 ± 0 3	−0 6	−0 7 ± 1 8	0 1 ± 0 4	0 8
MCV	4 0 ± 2 9	−4 6 ± 4 3	8 6	5 5 ± 4 1	2 3 ± 5 6	7 8
MCH	0 8 ± 1 0	−1 0 ± 1 1	1 8	−0 1 ± 0 6	−1 0 ± 1 1	0 9
MCHC	−0 7 ± 1 0	0 7 ± 1 0	−1 4	−2 5 ± 1 0	−0 2 ± 1 3	−2 3

0 5 and 1 0 ml packed cells The initial decrease in RCM was not statistically significant while the drop in hemoglobin hematocrit and number of erythrocytes was significant at a 5 per cent level This drop was partly due to a slight total loss of erythrocytes but mainly to hemodilution because the mean volume increased by 16 per cent when calculated on the basis of RCM and hematocrit

Fig 1 depicts the reticulocyte responses to ovine porcine and human GH The reticulocyte counts in all treated rabbits increased and in some up to 3 times the initial values The type of response was very similar to the reticulocyte response found follow-

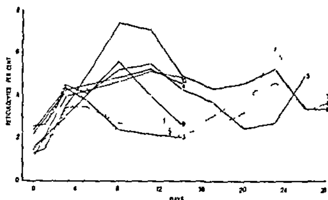


Fig 1 The reticulocyte response to human growth hormone ( ), ovine growth hormone (Prolactin) (— — — —) and porcine growth hormone (————) in rabbits

TABLE V Plasma erythropoietin levels in rabbits injected with growth hormone for varying periods of time

Rabbit no	Period of GH injections	Number of mice	Fe⁵⁹ per cent erythrocyte uptake ± S E: Hypoxia + transfusion induced polycythemic mice	Fe⁵⁹ per cent erythrocyte uptake ± S E: Transfusion induced polycythemic mice
GH 5—6	28 days	5	0 95 ± 0 15	
GH 7—8	28 days	4	0 88 ± 0 39	
GH 1	14 days	4	0 96 ± 0 32	
GH 3	14 days	5 (3)	0 35 ± 0 07	0 90 ± 0 46
GH 4	14 days	6		3 97 ± 2 10*
GH 5	14 days	2	0 50 ± 0 05	
GH 7—8	14 days	4	0 43 ± 0 24	
GH 15—16 17—18	3 days	5	2 03 ± 1 25	
GH 19	2 days	7	0 50 ± 0 02	
GH 13—14	2—24 hours	5	0 68 ± 0 14	
Saline controls		14	0 90 ± 0 21	
Saline controls		12		0 60 ± 0 14

ing erythropoietin administration, with no increase on the first day, a slight increase on the second and a definite increase on the third and fourth days The difference between the results obtained with the different GH preparations may be due to differences in dosage In principle the effect was the same In two rabbits receiving ovine GH the dose was doubled after 2 weeks of treatment causing a new rise in the reticulocytes

Table V shows the results of the erythropoietin assays in plasmas withdrawn following 2—24 hours and 2 3 14 and 28 days of GH administration There was no increase

TABLE VI Effect of daily porcine growth hormone (Somactone) injections on Fe^{59} erythrocyte uptake (72 hours uptake ± S E) in polycythemic mice

Daily doses	Number of mice	Hypoxia + transfusion induced polycythemic mice	Transfusion induced polycythemic mice
0 1 I U	3	0 75 ± 0 58	
0 5 I U	5	0 61 ± 0 25	
1 0 I U	5	1 31 ± 0 38	
1 0 I U	4		0 88 ± 0 24
3 0 I U	6	0 43 ± 0 21	
Saline controls	14	0 90 ± 0 21	
Saline controls	12		0 60 ± 0 14

TABLE VII Effect of daily porcine growth hormone (Somactone) injections on the erythropoietic response to a single dose of erythropoietin (ESF) in polycythemic mice measured as Fe^{59} erythrocyte uptake (± S E)

Material injected and dose of GH	Number of mice	Period of uptake after ESF (hours)	Hypoxia induced polycythemic mice	Transfusion induced polycythemic mice
ESF_a + GH 1 I U	4	48—120	16 1 ± 2 16	
ESF_a	3		11 3 ± 2 04	
ESF_b + GH 1 I U	19	48—120	37 7 ± 1 45	
ESF_b	10		39 0 ± 1 70	
ESF_c + GH 0 5 I U	7	0—72		2 84 ± 0 95
ESF_c	6			4 46 ± 1 49
ESF_c + GH 0 5 I U	5	48—96		11 0 ± 2 22
ESF_c	5			14 6 ± 3 86

The standard erythropoietin preparation has kindly been supplied by the Hematology Study Section of the U S Public Health Service As this special lot of erythropoietin (Lot 048) has lost more than usual activity by the storage in the cold the original figures in the unit are not given and the doses only referred to as a b and c

in Fe^{59} erythrocyte uptake in the recipient mice following administration of these plasmas expect from one rabbit (No 4) which increased Fe^{59} uptake slightly Table VI shows the Fe^{59} erythrocyte uptake in polycythemic mice injected with porcine growth hormone prior to and during the Fe^{59} administration Porcine growth hormone in doses from 0 1 to 3 0 I U on consecutive days did not increase Fe^{59} uptake in the recipients When erythropoietin was given in addition to porcine growth hormone there was no significant difference between the Fe^{59} uptake in this group and in a group

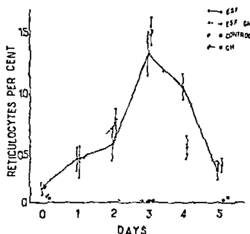


Fig 2 Effect of porcine growth hormone on the reticulocytes in transfusion induced polycythemic mice compared with saline injected controls and on the reticulocyte response to a single dose of standard erythropoietin (ESF) Each point represent the mean of 4—5 observations The brackets indicate 2 standard errors of the mean

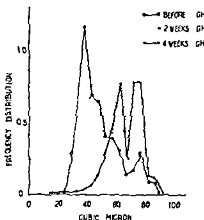


Fig 3 The red cell size distribution curves before (o————o) and after 14 ( ) and 28 (—————) days of porcine growth hormone administration in one rabbit (no 6)

receiving the same doses of standard erythropoietin alone (Table VII) The reticulocyte curves differed slightly but the data do not allow definite conclusions on this point (Fig 2)

Fig 3 shows the red cell size distribution curves before and following 14 and 28 days of GH administration in one rabbit The other three rabbits tested with this method all showed a shift to larger cells but not so marked as in the one shown on the figure This finding corresponds well with the observed increase on MCV following GH administration

The data from the haptoglobin determinations are not tabulated There was no difference between GH injected and control rabbits The red cell survival studies are also not more specifically documented Of 8 GH injected rabbits studied, 5 had apparent half lives between 8 and 14 days while the remaining 3 had apparent half lives between 15 and 18 days as the controls The red cell survival was tested both during and following the GH administration No difference was observed in mechanical fragility before and following GH administration

Discussion

The present study provides evidence that GH administration to intact rabbits causes peripheral reticulocytosis without increase in RCM, hemoglobin, hematocrit or erythrocytes and thus extends previous findings in hypophysectomized animals to intact animals In the present study the RCM dropped slightly (7 per cent) during the first days of GH treatment, increased thereafter to reach approximately the initial value at two weeks and remained at this level during the next two weeks The RCM/kg b w showed a slight rise after 2 weeks of GH administration, mainly because of weight loss in some of the rabbits Hemoglobin hematocrit and number of erythrocytes dropped significantly during the first days and remained below the pretreatment levels throughout the studies except for the hematocrit which approximated the initial values

The reticulocytes increased markedly from the second and third days of GH administration At the lowest doses of GH the reticulocytes levelled off following one to two weeks while they remained high throughout two weeks at higher doses The effect of dose on the reticulocyte level was demonstrated by showing that doubling the dose induced a second reticulocyte increase in 2 rabbits (no 7 and 8) in which the reticulocytes had fallen to the initial levels The reticulocytosis with concomitant drop in hemoglobin hematocrit and number of erythrocytes suggested that blood loss or destruction might be an explanation of the effect of GH in the intact rabbit This hypothesis could explain most of the effects of GH on erythropoiesis bone marrow hyperplasia, peripheral reticulocytosis drop in RCM and peripheral blood values and the increase in serum iron (Marinone and Meduri 1957) A possible hemolytic mechanism could be due either to a change in the external environment (plasma) or to a change in the metabolism of the red cells GH produces mobilization of fat and a marked increase on free fatty acids (Winkler *et al* 1964) Recent experiments have provided some evidence that hyperlipemia and rapid increase in free fatty acids may cause hemolysis (Schafiroff *et al* 1955 Greisman 1959) A similar mechanism could explain a possible hemolysis following GH administration Another possibility could be that GH might influence the red cell metabolism (glycolysis) thereby reducing their life span

The fall in hemoglobin (about 20 per cent) is, however, only partly explained by the fall in RCM (7 per cent) When the plasma volume is calculated on the basis of RCM and peripheral hematocrit, one may conclude that the mean plasma volume increased 16 per cent during the first three days of GH administration, and returned to the original level at 2 weeks The mechanism by which GH expands the plasma volume is unknown The present study gives some support to the hypothesis of Fruhman *et al* that the primary fall in the peripheral blood values is due to hemodilution, but also confirms Meineke and Crafts (1956) findings that GH administration does not increase the total RCM These effects seem to be the same both in hypophysectomized and intact animals

The further studies did not support the hypothesis of hemolysis as the mechanism for the GH effect on erythropoiesis The decrease in red cell survival was rather small, the mechanical fragility tests did not show any difference before and after GH treatment and serum haptoglobin did not change It must therefore be concluded that the initial drop in hemoglobin was due to hemodilution but the finding of a slight drop in total RCM may indicate that a small amount of erythrocytes was lost Hemodilution has not previously been found to stimulate erythropoiesis (Erslev 1959 and it is therefore more reasonable to search for the effect of GH in the bone marrow itself

The cell indices and the red cell size distribution curves indicate that GH causes an increase in red cell size The red cells produced seem to have a shortened life span both during and after GH administration The degree of shortening cannot be adequately demonstrated on the basis of the present studies because only a part of the red cell population studied had been produced during GH administration Increased red cell size and a shortened red cell survival is found following blood loss hypoxia hemolysis and following erythropoietin injections, i e in many states of increased red cell production These findings alone, do not therefore indicate that red cells produced following GH administration are abnormal cells However, since GH in contrast to erythropoietin does not increase the total red cell volume or hemoglobin its effect may be termed increased but ineffective erythropoiesis, and the cells produced are probably abnormal cells The reticulocyte curve following GH is very similar to that following erythropoietin with no increase on the first day This indicates that GH has no significant release effect on the reticulocytes

The plasma erythropoietin levels in the GH injected rabbits were studied after various periods of hormone administration One plasma withdrawn after 14 days of GH injection significantly (0 05 p 0 01) increased the Fe^{59} erythrocyte uptake in the recipient mice while none of the other tested plasmas did so It is therefore unlikely that the *effect of porcine growth hormone on erythropoiesis in the rabbit is mediated through* an increase of erythropoietin production One cannot totally disregard the one plasma which increased erythropoiesis in the recipients but this may sometimes also happen with normal plasma

Electrical stimulation of the hypothalamus caused a marked reticulocytosis in several of the stimulated rabbits and in eight rabbits an increase in RCM as well When the effect of GH is compared with the effect of hypothalamic stimulation it is evident that GH administration may give the same reticulocyte response as hypothalamic stimulation but not the increase in RCM It is therefore not likely that the effect of hypothalamic stimulation can be explained on the basis of increased GH production alone Four of the rabbits in the doubtful response group of the hypothalamic stimulation experiments did however have the same type of response as following GH injection i e marked *reticulocytosis without increase in RCM* One of these rabbits (K43) had an apparent red cell half life of 10 days Increased GH production cannot therefore be excluded as a factor in the influence of the hypothalamus on erythropoiesis

The polycythemic mouse offers a possibility of studying the effect of GH on differentiation from the hypothetical stem cell to erythroid cells and when GH is combined with erythropoietin it should be possible to study the effect on the later stages of proliferation and maturation GH alone did not increase the reticulocyte counts in the polycythemic mouse The Fe^{59} uptake was slightly higher in GH treated mice than in the controls but this may have been due to increased serum iron in the GH treated animals These findings indicate that GH does not increase differentiation from undifferentiated to erythroid cells. Combined GH and erythropoietin administration did not increase the 72 hrs Fe^{59} uptake more than erythropoietin alone The reticulocyte curves were however different in these two groups showing a more rapid decline in reticulocytes in the GH + erythropoietin treated group than in the erythropoietin alone group The finding was surprising and does not fit with a longer reticulocyte life span in the peripheral blood as the cause of the reticulocytosis following GH administration. Although not supported by the reticulocyte curves depicted in the present paper it *is most* likely that the effect of GH on erythropoiesis is not on the stem cell level but on the later stages of proliferation and maturation

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References

BRECHER, C
distributi
ENERBÄCK,
volume n
ERSLEY, A
1959 77
FISHER, J W, B L ROH C COUCH and W O NIGHTINGALE, Influence of cobalt, sheep

FRUHMAN, G J and A S GORDON, Effects of growth hormone and cortisol upon hemopoiesis *Acta haemat* (Basel) 1956 *15* 249—262
G

6—12
GREISMAN

HALVORSEN, S, Effects of ACTH on erythropoiesis in the rabbit *Acta physiol scand* 1963a *58* 30—39
HALVOR
of
HAL
ra
LI C
Chem 1954 *211* 555—557
LI C H, Hormones of the anterior pituitary gland Part II Melanocyte-stimulating and lactogenic hormones *Adv Protein Chem* 1957 *12* 269—317
MARTINONE G and MEDURI D, Influences hypophysaires sur le metabolisme du fer *Re Hemat* 1957 *12* 169—191
MEINEKE, H A and R C CRAFTS The effect of growth hormone on hematopoiesis in hypophysectomized adult female rats *Endocrinology* 1956 *59* 444—453
NYMAN, M, Serum haptoglobin Scand *J clin Lab Invest* 1959 Suppl 39
REMMELE, W, *Die Humorale Steuerung der Erythropoiese* Springer Berlin 1963
ROOS, P, H R FEVOLD and C A GEMZELL Preparation of human growth hormone by gel filtration
Sc

S
plasma *Proc Soc exp Biol* (N Y) 1955 *88* 224—226
S
A Effects of hypothalamic stimulation
1961 *13* 553—563
M EVANS Inability of growth hormone
Proc Soc exp Biol (N Y) 1952 *81*

WEINTRAUB A H A S GORDON and J F CAMISCOLI Use of the hypoxia-induced polycythemic mouse in the assay and standardization of erythropoietin *J Lab clin Med* 1963 *62* 743—752
WINKLER B R STEELE N ALTSZULER and R C DEBODO Effects of growth hormone on free fatty acid metabolism *Amer J Physiol* 1964 *206* 174—178

Acta physiol. scand. 1966. 66. 214–222

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Effects of Combined Hormone Therapy on Erythropoiesis in the Intact Rabbit

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Abstract

[illegible] increased both reticulocytes, red cell mass, red cell mass per kg body weight, hemoglobin, number of erythrocytes and hematocrit, and the effect following 2 weeks of administration was more marked than following electrical stimulation of hypothalamus. The end effect of combined hormone therapy on erythropoiesis cannot therefore be separated from the end effect of electrical stimulation of hypothalamus. There was, however, no significant increase in plasma erythropoietin levels in the group of rabbits receiving combined hormone therapy for 14 days when tested either in starved or in polycythemic mice. This finding may point to the existence of another operating mechanism in the rabbits receiving electrical stimulation of hypothalamus than in those receiving hormones.

In earlier studies we have found that electrical stimulation of the hypothalamus may stimulate erythropoiesis (Seip *et al.* 1961) and that hypothalamic lesions may interfere with the regulatory mechanism of erythropoiesis (Halvorsen 1961). The mechanism of these effects are, however, unknown. The possibility of an effect through the pituitary gland has not been excluded although neither ACTH nor combined pituitary hormone administration normalized the reticulocyte response to hypoxia in rabbits with lesions in the posterior hypothalamus. Neither did ACTH (Halvorsen 1963) nor growth hormone (GH) (Halvorsen 1965) have the same effect as hypothalamic stimulation when given to intact rabbits for 2 weeks. Electrical stimulation of the hypothalamus

may, however, increase the production of several pituitary hormones and it is the purpose of the present paper to report studies on the effect of combined pituitary and target organ hormones on erythropoiesis, and to compare this effect with the effect of hypothalamic stimulation

The effect of combined hormone therapy, both pituitary and target organ hormones has previously been studied mainly in hypophysectomized animals Fruhman and Gordon (1956) found that the effect of a combination of GH and cortisone on erythropoiesis was intermediate between the effect of the hormones given separately, i e a smaller reticulocyte increase than GH alone and a smaller increase in hemoglobin, number of erythrocytes and hematocrit than cortisone alone Meineke and Crafts (1957a)

lization both of the peripheral blood values and the bone marrow Shirakura (1961), on the other hand, did not observe normalization of the post hypophysectomy anemia when *thyroxin cortisone and testosterone were given to hypophysectomized rats*

In clinical medicine, therapy with a combination of corticosteroids and androgens has been a routine treatment in aplastic anemias since the original observation of Shahidi and Diamond (1959) With this treatment the anemia is corrected in several patients and in a few cases the hemoglobin is increased to levels above the normal (Shahidi 1963) The mechanism of this effect is largely unknown The androgens seem to be the most potent hormonal stimulator of erythropoiesis (Gardner 1962) and increase erythropoiesis in the recipients when given alone (Kennedy 1962) Naets (1964) observed that testosterone did not increase erythropoiesis in polycythemic mice, but *when given together with erythropoietin it increased erythropoiesis measured as* Fe^{59} uptake more than erythropoietin alone Fried *et al* (1964) also demonstrated that testosterone stimulated erythropoiesis in polycythemic mice

Since ACTH increased total RCM (Halvorsen 1963) and GH caused peripheral reticulocytosis (Halvorsen 1965) in intact rabbits, these two hormones were given to see whether this combination would mimic the effect of hypothalamic stimulation In a second series thyroxin and testosterone were added to ACTH and GH The target organ hormones were chosen instead of the tropic hormones mainly for practical reasons

Material and methods

Male

Ten

Porc

196

1963

sodium[3] and 1 mg testosterone proprionate[4] per kg b w per day for 2 weeks The control rabbits used in the present series are the same as described in the study on the effect of ACTH on *erythropoiesis (Halvorsen 1963*

[1] Somactone Ferring AB Malmo Sweden
[2] Jaton prolongatum Apothekernes Laboratorium Oslo Norway
[3] l thyroxin natrium pro injectione Nyegaard & Co A S Oslo Norway
[4] Testovin Nyegaard & Co A S Oslo Norway

TABLE I RCM RCM/kg body weight and hematocrit before and after a 2 week period of daily ACTH and GH (AG) injections

Rabbit no	RCM (ml)		RCM/kg (ml)		Ht	
	Before AG	After AG	Before AG	After AG	Before AG	After AG
1	45 8	50 4	18 8	19 8	—	—
2	37 0	33 2	18 5	15 4	—	—
3	55 3	58 1	19 5	21 9	46	48
4	55 9	66 2	18 4	23 2	43	46
5	72 9	77 2	20 0	22 1	46	43
6	77 2	73 7	23 7	23 4	48	43
7	55 0	64 6	20 0	22 0	48	16
8	55 0	55 3	24 0	22 5	45	43
10	59 4	59 6	21 8	22 2	46	46

TABLE II Mean of the differences between the post and pre treatment values (± S E) The differences of the means between the ACTH and GH (AG) treated and the control groups are tabulated in the third column

	AG (9)	Control (10)	AG − Control
RCM ml	2 7 ± 1 68	1 8 ± 2 2	0 9
RCM/kg ml	0 9 ± 0 78	−0 4 ± 0 5	1 3
Ht %	−1 2 ± 0 86	−0 9 ± 1 1	−0 3
Hgb g	−0 6 ± 0 37	0 01 ± 0 3	−0 61
Ery s mill	−0 36 ± 0 14	0 19 ± 0 3	−0 55
MCV	3 6 ± 2 63	−4 6 ± 1 3	8 2

Before and after the hormone administration red cell mass (RCM) was determined with the Cr^{51} method as previously described (Halvorsen 1963) At the same time reticulocytes, hemoglobin red blood cells and hematocrit were determined using the methods previously described The reticulocytes were counted every third to fourth day and hemoglobin red blood cells and hematocrit were also determined on the third day of hormone administration

Plasma from the rabbits was withdrawn at the end of the experimental period and tested for erythropoietin in starved mice (Halvorsen ... mice as described by Weintraub *et al* (1963) ... given 0 7 ml homologous washed red cells on ... mice of the 5NMRI BOM strain weighing between ...

Results

Table I shows the RCM RCM kg b w and hematocrit in 10 rabbits receiving GH—ACTH for 2 weeks In 2 rabbits there was an increase in RCM in the others there was no or only a slight increase Table II summarizes the data from this group of

Fig 1 Fig 2

Fig 1 The upper figure shows the mean reticulocyte response to ACTH — GH (AG) injection in 10 intact rabbits compared with the reticulocyte values in 10 control rabbits The lower figure shows the mean hemoglobin and hematocrit before and after 3 and 14 days of ACTH — GH injections The brackets indicate 2 standard error of the mean

Fig 2 [illegible]

rabbits expressed as the mean of the differences between the post- and pre treatment values There was no significant change in RCM or RCM/kg b w although the RCM increased slightly more than in the controls There was no significant difference in hemoglobin, erythrocytes or hematocrit before and after the hormone administration As depicted in Fig 1 however these parameters dropped during the first few days of hormone administration The MCV increased while the MCH and MCHC did not change Table II summarizes the data from the 4 rabbits receiving GH — ACTH for 4 weeks The increase in RCM in these 4 rabbits closely resembled the control rabbits. In these rabbits the hemoglobin number of erythrocytes and hematocrit levels decreased

Fig 1 depicts the reticulocyte response during GH — ACTH administration The reticulocytes definitely increased during the first days and leveled off towards the end of the observation period The figure further shows the hemoglobin and hematocrit levels before and following 3 and 14 days of hormone administration indicating a drop in the values during the first days

The 8 rabbits receiving GH — ACTH — thyroxin — testosterone all lost weight the mean weight loss being 240 g One rabbit died during the period The RCM, RCM/kg b w and hematocrit of the remaining 7 rabbits are shown in Table III The RCM increased in all animals and the increase was significantly higher than in the control group The relative increase in RCM kg b w was even greater but as the

TABLE III RCM, RCM/kg body weight and hematocrit before and after a 2 week period of daily GH — ACTH — thyroxin — testosterone (GATT) injections

	RCM (ml)		RCM/kg (ml)		Ht	
Rabbit no	Before GATT	After GATT	Before GATT	After GATT	Before GATT	After GATT
1	50 1	62 6	17 9	27 2	40	47
2	51 2	57 1	19 6	24 0	40	51
3	47 3	71 5	18 8	31 0	40 5	48
5	62 3	71 2	24 4	28 8	41	52
6	49 9	56 7	19 2	22 4	44	48 5
7	54 7	59 4	20 6	24 0	42	50
8	47 8	56 0	19 8	27 2	42	50 5

TABLE IV Mean of the differences between the post and pre-treatment values (± S E) The differences of the means between the GH — ACTH — thyroxin — testosterone (GATT) treated and the control groups are tabulated in the third column. In this and the following tables one asterisk indicates $0\ 05 > p > 0\ 01$, two asterisks $0\ 01 > p > 0\ 001$ and three asterisks $p < 0\ 001$

	GATT (7)	Control (10)	GATT — Control
RCM ml	10 2 ± 2 53	1 8 ± 2 2	8 4*
RCM kg ml	6 3 ± 1 29	−0 4 ± 0 5	6 7***
Ht %	8 2 ± 0 86	−0 9 ± 1 1	9 1***
Hgb g	2 3 ± 0 42	0 01 ± 0 3	2 29
Erys mill	1 13 ± 0 16	0 19 ± 0 3	0 94*
MCV	0 14 ± 1 97	−4 6 ± 4 3	4 74

animals lost weight this figure may give a false impression of the magnitude of the response Table IV summarizes the data from this group Both hemoglobin number of erythrocytes and hematocrit increased significantly while the cell indices did not show significant changes

Fig 2 depicts the reticulocyte response in this group of rabbits and all showed a moderate rise The hemoglobin decreased slightly during the first days of hormone administration as in all groups receiving GH but increased thereafter markedly as shown in the figure

Table V and VI show the results of the plasma erythropoietin assays None of the tested plasmas withdrawn following 2 weeks of hormone therapy increased Fe^{59} erythrocyte uptake in the starved or polycythemic mice Plasma from the rabbit with the most marked increase in RCM following GH—ACTH—thyroxin—testosterone (GATT

TABLE V Per cent Fe^{59} erythrocyte uptake (72 hrs) in starved mice injected with plasma (0 4 ml × 2) from 14 days GH — ACTH — thyroxin — testosterone (GATT) treated rabbits and with control plasma

Material injected	Number of mice	Fe^{59} % uptake ± S E	Diff
Control plasma	16	23 2 ± 2 0	
GATT 1	5	23 7 ± 3 0	0 5
GATT 3	10	10 0 ± 2 2	−13 2***
GATT (5 + 8)	10	19 3 ± 3 0	−3 9
GATT (6 + 7)	6	20 4 ± 3 6	−2 8

TABLE VI Plasma erythropoietin levels in rabbits injected with ACTH — GH (AG), GH — ACTH — thyroxin — testosteron (GATT) for 14 days and with GATT for 2 days

		Fe^{59} erythrocyte uptake ± S E	
Material injected	Number of mice	Hypoxia induced polycythemic mice	Hypoxia + transfusion induced polycythemic mice
Saline	19	2 8 ± 0 8	
GATT 1	5	1 1 ± 0 5	
GATT 2	5	2 2 ± 0 8	
GATT 3	4	3 3 ± 2 3	
Saline	14		0 9 ± 0 2
GATT 1 + 3	2		1 1 ± 0 1
GATT 5 + 8	9		0 8 ± 0 2
GATT 6 + 7	5		0 3 ± 0 1
AG 1 + 2	1		0 3
AG 5 + 6	1		0 6
AG 7 + 8 + 10	5		2 1 ± 1 3
GATT 2 days	7		2 8 ± 1 2*

3) reduced the Fe^{59} erythrocyte uptake significantly in the starved mice Plasma withdrawn on the third day from a rabbit receiving GATT on first and second days slightly increased the Fe^{59} uptake in polycythemic mice

Discussion

The hormonal control of erythropoiesis has been documented in experimental and clinical studies and has been reviewed by several authors (Gordon 1954 and 1959 Linman and Bethell 1961 Evans *et al* 1961 and Remmele 1963) The mechanism of

the effect of the individual hormones, the interaction of the hormones, the interaction of the hormones with erythropoietin and the significance of the hormones in the steady state of erythropoiesis are, however, still debated In the present study the effect of combinations of hormones has been investigated

Combined GH — ACTH administration did not change erythropoiesis significantly and had less effect than the individual hormones given alone in the same doses GH — ACTH increased total RCM slightly but less than ACTH alone, and also increased the reticulocytes but less than GH alone The hemoglobin, number of erythrocytes and hematocrit decreased definitely as with GH alone during the first days due to hemodilution, but reached approximately the initial levels by 2 weeks The present study thus confirms, and extends to intact animals previous studies in hypophysectomized animals in which GH and cortisone seemed to counteract each other in the effect on erythropoiesis (Fruhman and Gordon 1956) The mechanism of this interaction is unknown but the antagonism between GH and cortisone is well known from other organs (Soyka and Crawford 1964)

The combination of GH — ACTH — thyroxin — testosterone was used to see whether a more general hypersecretion of anterior pituitary hormones might mimic the erythropoietic response to electrical stimulation of the hypothalamus It may be argued that it is doubtful whether the effect of the target organ hormones can be compared directly with the effect of the tropic hormones The increase in thyroid hormones following injections of thyrotropin is however, well documented, and the gonadotropins probably also increase testosterone production (Eik Nes 1964) Using the target organ hormones instead of the tropic hormones the dose will be more likely to be too large than too small

This combination of hormones had a marked stimulatory effect on erythropoiesis. Total RCM RCM/kg b w number of erythrocytes and hematocrit increased significantly The increase in RCM/kg b w may give a false impression of the magnitude of the erythropoietic response because the rabbits lost weight The red cell indices did not change significantly in this series of animals

The plasma erythropoietin levels were not elevated in these rabbits and plasma from the rabbit with the most marked increase in RCM inhibited the erythropoiesis in the starved mice The question of erythropoiesis inhibitors is unsolved although the studies of Krzymowski *et al* (1962) and Reynafarje *et al* (1964) indicate their existence The finding of an inhibitory effect of plasma following hormone administration suggests at least that the increase in erythropoiesis has not been mediated through erythropoietin as the main mechanism If the increased erythropoiesis was the result of an increased erythropoietin production one would also have expected an increase in red cell size (MCV)

Thyroxin and testosterone have not been tested individually by us so it is difficult to define which hormone has been the most effective in this combination of hormones Gardner (1962) states that testosterone is probably the most effective non specific stimulator of erythropoiesis and it is likely that this hormone has been most effective in this study In view of recent work by Naets (1964) and Fried *et al* (1964) the effect of testosterone can no longer be considered to be non specific with its influence on oxygen consumption alone but there is also a direct effect on the erythroid cells. From their work on the interaction of hormones with erythropoietin Evans *et al* (1964) concluded that several erythropoietically active pituitary hormones and erythropoietin interact in an additive manner to maintain normal erythropoiesis These and previous

findings in hypophysectomized and intact animals indicate that the hormones are also of fundamental importance for the normal steady state erythropoiesis

The primary purpose of this study was to compare the effect of combined hormone administration with the effect of electrical stimulation of the hypothalamus GH — ACTH did not increase RCM and it is unlikely that these two hormones alone were the cause of the effect on erythropoiesis following hypothalamic stimulation

The combination of GH — ACTH — thyroxin — testosterone did, however, cause the same or a higher increase in total RCM and RCM/kg b w than hypothalamic stimulation, and also increased the peripheral blood values significantly, in contrast to the hypothalamic stimulation which only caused a slight increase in hematocrit but no increase in hemoglobin or number of erythrocytes The reticulocyte response was lower in the hormone treated group than in the positive response group, but the type of response was essentially the same with increases during the first week and a fall during the next week The MCV increased markedly following hypothalamic stimulation while there was no change following the combined hormone administration Following electrical stimulation of hypothalamus we found higher plasma erythropoietin levels than in control rabbits while no increase, and rather an inhibitory effect on Fe^{59} erythrocyte uptake in starved mice was found with plasma from rabbits injected for 14 days The slightly increased Fe^{59} uptake in polycythemic mice injected with plasma from one rabbit receiving GATT for 2 days may indicate that erythropoietin initially was increased

Although both hormone treatment and electrical stimulation of the hypothalamic region caused reticulocytosis and RCM increase this does not prove that the operating mechanism has been the same The differences in the erythropoietin response may point to separate mechanisms It should also be remembered that the doses used in the combined hormone studies probably exceeded physiological doses considerably It must, however be concluded that it is difficult to evaluate a possible extra pituitary effect from the hypothalamic region on erythropoiesis in stimulatory experiments unless the studies are performed in hypophysectomized animals This fact has been overlooked in many studies dealing with the central nervous system control of erythropoiesis and the present finding further stresses the caution needed in interpreting the effect on erythropoiesis of procedures which interfere with body homeostasis as markedly as most procedures on the central nervous structures

References

[illegible] The mouse with hypoxia induced erythremia [illegible] *Med* 1962 *60* 846—859
[illegible] retion of steroids by the testis and ovary [illegible]
[illegible] Erythropoietic response to calorigenic [illegible]
[illegible] Interaction of the ESF with calorigenic hormones *Endocrinology* 1964 *75* [illegible]
FRIED W R DEGOWIN R FORDE and C W GURNEY Erythropoietic effect of testosterone in [illegible] (N Y) 1964 *117* 839—842
FRUH [illegible] with hormone and cortisol upon hemopoiesis [illegible]
GARD [illegible] *with non specific stimulation of erythropoiesis in man Erythropoiesis* Ed L. O Jacobson and M Doyle Grune & Stratton New York 1962 pp 262—269

GORDON A S, Endocrine influences upon the formed elements of the blood and bloodforming organs *Recent Progr Hormone Res* 1954 *10* 339—391

GORDON, A S, Hemopoietine *Physiol Rev* 1959 *39* 1—40

HALVORSEN, S, Plasma erythropoietin levels following hypothalamic stimulation in the rabbit *Scand J clin Lab Invest* 1961 *13* 564—575

HALVORSEN, S Effects of ACTH on erythropoiesis in the rabbit *Acta physiol scand* 1963 *58* 30—39

HALVORSEN S, Effects of hypothalamic lesions on the erythropoietic response to hypoxia in rabbits *Acta physiol scand* 1964 *61* 1—19

HALVORSEN, S, Effects of growth hormone on erythropoiesis in the intact rabbit and the polycythemic mouse *Acta physiol scand* 1966 *66* 203—213

KENNEDY, B J Stimulation of erythropoiesis by androgenic hormones *Ann int Med* 1962 *57* 917—936

... in the

... rning

field Illinois 1960

MEINEKE, H A and R C CRAFTS Further observations on the effect of cortisone and thyroxine on the blood picture of hypophysectomized rats *Blood* 1957a *12* 11—23

MEINEKE H A and R C CRAFTS Effect of combined thyroxine — cortisone — growth hormone therapy on hematopoiesis in hypophysectomized rats *Proc Soc exp Biol (N Y)* 1957b *96* 74—79

R

R

649—650

SEIP M S HALVORSEN, P ANDERSEN and B R KAADA Effects of hypothalamic stimulation on erythropoiesis in rabbits *Scand J clin Lab Invest* 1961 *13* 553—563

SHAHIDI N T and L K DIAMOND, Testosterone-induced remission in aplastic anemia *Am J Dis Child* 1959 *98* 293—302

S ... of erythrocytes in testosterone- ... *J Lab clin*

... nd endocrine organs *Acta haemat jap* 1961 *24* 536—544

SOYKA L F and J D CRAWFORD Antagonism of human growth hormone (HGH) by cortisone *J Pediat* 1964 *65* 1115—1117

WEINTRAUB A H A S GORDON and J F CAMISCOLI Use of the hypoxia induced polycythemic mouse in the assay and standardization of erythropoietin *J Lab clin Med* 1963 *62* 743—752

Acta physiol. scand 1966 66. 223—225

From the Department of Pharmacology, University of Goteborg Sweden

Dopamine in Human Urine during Muscular Work

By

JAN HÄGGENDAL and BENGT WERDINIUS

Received 28 June 1965

Abstract

Häggendal, J and B Werdinius *Dopamine in human urine during muscular work* Acta physiol scand 1966 *66* 223—225 — In four healthy men the urinary excretion of total (free and conjugated) adrenaline and noradrenaline rose during hard muscular work while dopamine and homovanillic acid were unchanged or even decreased somewhat The source of urinary dopamine is discussed

During muscular work the levels of the catecholamines noradrenaline (NA) and adraneline (A) increase in urine (Euler and Hellner 1952) and in blood plasma (*e g* Vendsalu 1960 Haggendal 1963 a) The increase includes both the free compounds and the total (free + conjugated) amounts of the substances obtained after acid hydrolysis At moderate muscular work chiefly the NA level is raised which is due to increased activity of the sympathetic nervous system At very hard work and or psychical stress the A level is also increased due to adrenomedullary secretion

Dopamine (DA) is the immediate precursor in the synthesis of NA and A but recently neurons have been found which probably have DA as the transmitter e g in the retina (Malmfors 1963 Malmfors and Haggendal 1963) and in the central nervous system (Andén *et al* 1964)

DA is normally present in the urine in both free and conjugated form and in amounts larger than those of free and conjugated NA and A respectively So far the presence of DA in blood plasma does not seem to have been established the methods available for DA assay are at present not so sensitive as those for NA and A

The source of DA in urine remains to be discovered Since it cannot be excluded that it is derived from the adrenergic nerves where it is generally believed to be formed as an intermediate in the synthesis of NA it was thought to be of interest to follow the urinary DA during muscular work

In this preliminary report we have investigated the amounts of urinary NA A and DA after acid hydrolysis before and during heavy muscular work To get a more complete picture we have also included the main DA metabolite homovanillic acid (HVA) in some of the experiments

TABLE I

Subject	Sum of total (free+conjugated) A and NA (ng/min): Before start 'at rest'	Work period I	Work period II	Total DA (ng/min) and total HVA (µg/min): Before start 'at rest' DA	HVA	Work period I DA	HVA	Work period II DA	HVA
1	167	300	524	1 366	5 8	615		768	
2	74 NA 30% A 70%	115 NA 88% A 12%	447 NA 83% A 17%	234		120		258	
3	59 NA 66% A 34%	294 NA 69% A 31%	253 NA 67% A 33%	246	3 6	145	3 1	153	2 6
4	144 NA 82% A 18%	134 NA 63% A 37%	572 NA 42% A 58%	968	7 1	747	6 8	648	4 5

Four healthy men about twenty years old and in good physical health were random chosen for investigation in a very hard military cross country race The men had volunteered for special service and used to take themselves out almost completely Urine samples were collected in three portions from every man 1) During a 45 min period before the start (at rest 2) During the period 15 min before the start and until half the distance was run work period I) and 3) During the rest of the distance and until 15 min after the end of the run (work period II) The whole race took about 2 1/2 hrs Perchloric acid was immediately added to the samples After acid hydrolysis (in N perchloric acid for 15 min the total (free + conjugated) NA and A was estimated according to Häggendal 1963 total DA according to Carlsson and Waldeck (1958) modified by Carlsson and Lindqvist 1962 and total HVA according to Werdinius (1966

The results are given in Table I In the first subject differentiation between NA and A was not performed

In agreement with earlier investigations the levels of NA and A increased during work

The DA levels differed from this picture All the values of total DA except one were somewhat lower during the work periods than before The DA metabolite, HVA did not show any certain changes perhaps there was a slight decrease

If the source of the DA in the urine is the adrenergic neurons an increased activity in these neurons did apparently not cause an increased release of DA in proportion to the release of NA However it does not seem unlikely that the urinary DA is derived from other structures than the adrenergic nerves and that these structures are not activated at muscular work It may be recalled that DA has been detected in mast cells from certain mammals Bertler *et al* 1959, Falck *et al* 1964, Adams Ray *et al* 1965

During the physical activity the proportion between free and conjugated catechol amines was changed (Haggendal 1963 b) The changed circulatory state in the body might influence the metabolism of the compounds

To elucidate these problems the pattern of free and conjugated catecholamines and their metabolites must be followed in closer detail

This work has been supported by grants from the Swedish State Medical Research Council (Nr F-0166-B A and 14× 166 02) For technical assistance we are indebted to Miss Anita Wallin

References

ADAMS-RAY J A DAHLSTROM K FUXE and J HÄGGENDAL, Formation and storage of dopamine in hamster mast cells following the administration of dopa *J Pharm Pharmacol* 1965 *17* 252—253

ANDEN N E, A CARLSSON A DAHLSTROM K FUXE N-Å HILLARP and K LARSSON Demonstration and mapping out of nigro neostriatal dopamine neurons *Life Sci* 1964 *3* 523—530

BERTLER Å, B FALCK, N Å HILLARP E ROSENGREN and A TORP, Dopamine and chromaffin cells *Acta physiol scand* 1959 *47* 251—258

CARLSSON A and B W... method for the de...
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FALCK B T NYSTEDT, E ROSENGREN and J STENFLO Dopamine and mast cells in ruminants *Acta pharmacol* (Kbh) 1964 *21* 51—58

HÄGGENDAL J, An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues *Acta physiol scand* 1963 a *59* 242—254

HÄGGENDAL, J, The presence of conjugated adrenaline and noradrenaline in human blood plasma *Acta physiol scand* 1963 b *59* 255—260

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VENDSALU A, Studies on adrenaline and noradrenaline in human plasma *Acta phy l scand* 1960 *49* Suppl 173

WERDINIUS B A flourometric method for determination of homovanillic acid in urine 1966 To be publ

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From the Institute of Physiology, University of Oslo, Norway

Vasoconstriction in Isolated Blood-Perfused Rabbit Lungs and its Inhibition by Cresols

By

A. HAUGE, P. K. M. LUNDE and B. A. WAALER

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Abstract

HAUGE, A., P. K. M. LUNDE and B. A. WAALER. *Vasoconstriction in isolated blood-perfused rabbit lungs and its inhibition by cresols.* Acta physiol. scand. 1966. 66. 226—240. Perfusion of isolated rabbit lungs with heparinized whole blood was complicated by the development of a very marked increase in pulmonary vascular resistance (PVR), which occurred in the course of from 10 min to 2 hr. *This increase in PVR occurred also when nearly all thrombocytes had been removed from the perfusate.* Inhibition of the vasoconstrictor action of noradrenaline, 5-hydroxytryptamine, histamine or acetylcholine did not prevent the development of the increase in PVR. Plasma kinins, which cause marked vasoconstriction in this preparation, were not [illegible] PVR. The increase in [illegible] e creal [illegible] Adren [illegible] caused vasoconstriction in the preparation. Their vasoconstrictor effect, which increased very markedly during the first part of the perfusion, was inhibited by tri-cresol. Also the vasoconstrictor effect *of noradrenaline* was reduced by tri-cr[illegible] on the [illegible] spontaneously increasing PVR, tri-cres[illegible] on [illegible] relationship between the increase in P[illegible] seems likely.

Isolated perfused lung preparations are widely used in physiology and pharmacology. They are often taken from cats and dogs, species which seem to yield good preparations. Whole blood has been successfully used as perfusate in such lungs by several investigators. Isolated perfused rabbit lungs have also been employed in various fields of research, but many investigators working with such preparations seem to have preferred perfusates other than blood (Born 1954, Koga 1954, Engelberg and DuBois 1959, Lecomte and Troquet 1960).

We attempted to perfuse isolated rabbit lungs with whole blood, because no other perfusate seemed to have the same ability to prevent or delay edema formation. It

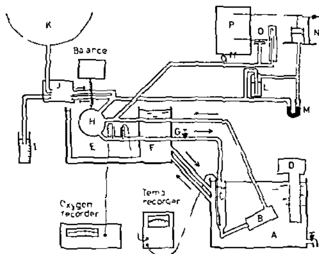


Fig 1 *Diagram of the perfusion arrangement*
A and F thermostatically controlled waterbaths B head of perfusion pump C blood reservoir D thermostat with water pump E organ chamber (usually covered) G clamp

Q time and signal markers For further explanation see text

was found, however, that the blood perfused preparations suffered from the disadvantage that the pulmonary vascular resistance progressively increased to a marked degree The investigation to be described gives some information about the nature of this increase in resistance, and presents evidence that it can be efficiently counteracted by the addition of small doses of tri cresol

Methods

The time between the arrest of the animals own circulation and the start of the perfusion was usually between 11 and 20 min in most experiments about 15 min The preparation taken out consisted of both lungs with the trachea the heart and the pulmonary vessels The caval veins and the aorta were ligated As much as possible of the other mediastinal structures (fat

tration of about 30 i u ml blood

The blood from the two donors was mixed and kept in a closed glass container at room temperature until the start of the perfusion. Compatibility tests were carried out in several experiments between the bloods of the two donors and between their red blood cells and the serum of the animal from which the lungs were taken. Agglutination was seen only once in 30 tests.

Perfusion with 180 ml of autologous blood could also be carried out. A reduced external perfusion circuit was then used, the animal being exsanguinated just before the lungs were taken out.

Perfusion. The technique described by Daly and Waaler (1961) for the perfusion of isolated ... rangement ... e pulsatile ... D/S) pump ... was almost ... al pressure (LAp) was usually kept constant throughout an experiment at a level between 3 and 6 cm of water.

The use of a cross-coupling arrangement for the inflow and outflow tubings made it possible to reverse the direction of the blood flow to a left atrial inflow situation, without arresting the circulation.

was done at the end of the experiment by placing suitable weight loads on the prepara...

Left atrial outflow measurements were done intermittently by collecting the blood in a graded cylinder for periods of from 10—30 sec.

Cleaning of the equipment was carried out mainly as described by Allison, Daly and Waaler (1961). The plastic parts were cleaned with a 1 per cent solution of benzalcon, rinsed ten times with distilled water and dried at 45° C.

Perfusate temperature was recorded in the venous reservoir and in the venous outflow tubing (Fig. 1), a multichannel YSI telethermometer, Model 44 YA (Yellow Spring Instrument Co.) being used. Temperature adjustments could be obtained by altering the setting of the water ... erbath ... essure ventilation with a ... lead" pump (C. F. Palmer ... ent described by Konrett and Rossler (1940). Peak inspiratory pulmonary pressure (IPp) was allowed to reach 10 cm of water and expiratory pulmonary pressure (EPp) was kept at 1.5—2 cm of water. In some experiments the ventilation gas was moistened by passing it through water in a bubbling chamber.

Blood platelets were counted according to the method of Brecher and Cronkite (1950).

Hematocrit values were estimated using an International micro-capillary centrifuge, model MB.

Hemolysis. After centrifugation at 2 000 × *g* for 5 min the hemoglobin content of the blood plasma was determined as cyanmethemoglobin at 540mμ in a Zeiss spectrophotometer, model PMQ II, using the method of Drabkin (1935) with some minor modifications.

pH of the perfusate was measured using the Astrup Micro equipment, Type AME c from Radiometer.

Plasma electrolytes (Na, K) were estimated with an Eppendorf flame photometer.

Plasma glucose was determined by the glucose oxidase method of Huggett and Nixon (1957) with some modifications recommended by the producer of the reagents (Kabi, Stockholm, Sweden).

... into the pulmonary arterial tubing near the ... ns of drugs were performed ... B. Braun (Melsungen, W. ... an infusion volume of 0.5 ml/min.

Some drugs (inhibitors) were added to the blood reservoir in order to obtain rapid mixing with the perfusate.

TABLE I Comparison between blood flow through 53 isolated perfused rabbit lungs and the cardiac output of living rabbits (as given by Krayenbuhl, Kako and Luthy (1964))

Mean values for 53 perfusion experiments			Mean value for living rabbits (Krayenbuhl *et al* 1964)
PAp (cm of water)	Blood flow through lungs (BF) (ml/min)	$\frac{\text{BF}}{\text{body weight}}$ $\left(\frac{\text{ml}}{\text{min kg}}\right)$	$\frac{\text{Cardiac output}}{\text{body weight}}$ $\left(\frac{\text{ml}}{\text{min kg}}\right)$
20 7 (S D 2 5)	234 (S D 76)	78	95

The calculations are based on values from the initial phase (first 60 m n) of 53 typical and successful experiments with perfusion of isolated rabbit lungs with homologous, heparinized blood at constant volume inflow The lowest value for the pulmonary arterial pressure (PAp) has been taken from each experiment The mean weight of the animals from which the lung preparations had been taken was 3 0 kg

List of drugs used

[illegible]

Inhibitors

As [illegible] (Deseril® Sandoz A. G) [illegible] & Baker) As inhibitor of [illegible] (Regitin® Ciba Societe [illegible]

Antibiotics

Penicillin G (Crystalline sodium penicillin G Glaxo) Semisynthetic penicillin — Cloxacillin (3-(2-chlorophenyl 5-methyl isoxazolyl sodium penicillin) Ekvacillin® Norsk Astra) Dihydrostreptomycin (Dihydrostreptomycin Glaxo

Results

During the first part of the perfusion a gradual reduction in pulmonary vascular resistance (PVR) was usually seen Flow rates compatible with the values for cardiac output in the resting rabbit (Krayenbuhl Kako and Luthy 1964 were obtained in most preparations (Table I

Within a period of from 10 min to 2 hr after the start of the perfusion there almost invariably occurred a marked increase in PVR. This increase was seen also when autologous blood only was used as perfusate. Humidification of the ventilation gas did not prevent the PVR increase. Preliminary bacteriological tests of the perfusate showed that a bacteriaemia might develop in the course of a perfusion. Addition to the perfusate of large doses of a semisynthetic penicillin (20 mg/100 ml of Ekvacillin®) or of a combination of a penicillin (5 mg/100 ml of penicillin G added every second hr) and of dihydrostreptomycin (4 mg/100 ml) did not prevent the increase in PVR. Some growth of microorganisms resistant to these antibiotics was, however, difficult to avoid.

The rate of rise in resistance varied considerably from one preparation to another and would sometimes increase progressively towards an almost complete closure of the pulmonary vascular bed. A reduction of the blood flow by altering the pump setting could then only delay this result. In preparations where the development was less marked, the increased PVR would last for several hours or for the remaining part of the perfusion.

The increase in PVR was sometimes initiated or accelerated by an extra inflation of the lungs or by reversing the direction of the perfusion. Usually the increase in resistance would start without any apparent provocation of the preparation.

It became essential for us to be able to control the increase in vascular resistance. Two main types of mechanisms might be responsible for this increase. Either a severe pulmonary vasoconstriction took place, or there occurred marked rheological changes in the blood perfusate. For several reasons the thrombocytes attracted our attention in this connection. Firstly thrombocyte aggregation with plugging of small pulmonary vessels might occur (Waalkes and Coburn 1959). Secondly vasoactive substances might be liberated from the thrombocytes in connection with a possible aggregation or disintegration of these elements. Rabbit thrombocytes are also known to have a high content of 5-hydroxytryptamine and histamine (Humphrey and Jaques 1954).

Number of circulating thrombocytes in the perfusate. The thrombocyte count in blood from the ear artery in 10 intact rabbits was found to range from 300 000 to 550 000 per mm³. During collection and storage of perfusate blood a considerable fall in the number of thrombocytes occurred. This fall will not be discussed here, but is under further investigation.

The number of thrombocytes in the blood just at the start of perfusion was found to vary between 30 000 and 250 000 per mm³. In the course of a perfusion there occurred a further gradual decline in the number of circulating thrombocytes. No relationship between the number of circulating thrombocytes on the one hand and the tendency towards a marked increase in PVR on the other could be seen. However, Furthermore removal of almost all thrombocytes from the perfusate in 4 expts by filtering the blood through glass wool (Pyrex Brand Wool 3 g/100 ml of blood) and discarding the first 50 ml of blood coming out from the lungs did not prevent the gradual and marked increase in PVR. After such a filtration the perfusate contained about 10 000 thrombocytes per mm³ or less.

Mechanical plugging of the vascular bed by thrombocyte aggregates or liberation of vasoactive substances from thrombocytes alone could thus apparently not explain the marked increase in PVR in this preparation. Vasoconstrictor substances might, however, be liberated into the blood stream also from sources other than the thrombocytes.

Effects of various inhibitors. Plasma kinins, noradrenaline, 5-hydroxytryptamine, acetylcholine and usually also histamine all cause pulmonary vasoconstriction in our

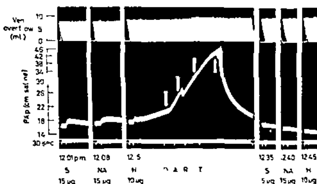


Fig 2 *Effects of inhibitors Deseril®, Anthisan® and Regitin® and of tri-cresol on increasing pulmonary vascular resistance (PVR)*

started at 11 29 a m Blood flow (BF) through preparation 260 ml min Left atrial pressure (LAp) 4 5 cm H_2O Tidal volume (TV) decreased from 42 to 40 ml during the period illustrated Vent overflow = ventilation overflow PAp = pulmonary arterial pressure

preparation (Hauge, Lunde and Waaler 1965) The possible role of these various substances for the increase in PVR was evaluated by addition of various inhibitors

Complete inhibition of the vasoconstrictor effect of injected doses of 5 hydroxytryptamine, histamine or catecholamines could be obtained through additions to the perfusate of adequate doses of Deseril®, Anthisan® or Regitin® respectively Such additions or atropinization of the preparation (1 mg of atropine added to the perfusate) did not however, prevent the development of an increased PVR in our preparation Once the increase in PVR had started, the addition of the various inhibitors similarly did not stop this development although Anthisan® caused a moderate short lasting reduction in PVR (Fig 2)

The possible role of plasma kinins for the increase in PVR was evaluated by analyzing samples of in and outflowing blood for their content of kinins Samples were taken directly into ethyl alcohol of 70° C dried, redissolved (Hamberg and Rocha e Silva 1957) and tested on the isolated rat uterus preparation Significant plasma kinin activity was detected neither in blood samples taken during periods of high and increasing PVR nor in samples taken during low and stable PVR

The effect of tri cresol and related compounds Among the various chemical compounds tested, tri-cresol was found to influence the increasing PVR markedly when added to the perfusate Fig 2 shows the effect of an injection into the pulmonary arterial tubing of 2 mg of tri cresol (at T in a preparation with a rapidly rising PAp and where previous additions of Deseril®, Anthisan® and Regitin® had not influenced the development The pressure rise was arrested and a gradual reduction in PVR followed

When the increase in PVR was initiated by a reversal of the perfusion (blood now entering the lungs through the pulmonary veins the effect of tri-cresol addition was equally marked (Fig 3 After a short period of normal inflow a new reversal of the perfusion could be done without any progressive increase in PVR



from 37 to 36 ml during the period illustrated Abbreviations as in Fig 2

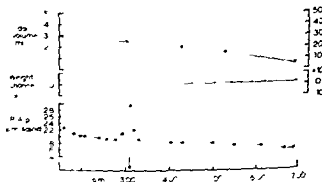


Fig 4 The development of tidal volume weight of preparation and pulmonary arterial p... perfusion where tri-cresol was added
IPL, rabbit ?9 kg Injection into the pulmonary arterial tubing of 2 mg of tri-cresol at arrow Perfusion started at 1 1? p m BF 140 ml min LAp 4 cm H_2O Abbreviations as in Fig 2

The effect of a 2 mg addition of tri-cresol usually lasted for several hr often for the rest of the perfusion period Occasionally a second and a third addition was found necessary to control the PAp level

Tri-cresol is a mixture of the three monocyclic isomers o-cresol p-cresol and m-cresol A series of experiments was carried out in which the effect of each of the three isomers was tested separately M-cresol was found to have a very moderate effect on the increase in PVR whereas o- and p-cresol were much more effective and also apparently equally potent In some preparations tri-cresol had a marked effect, whereas previous additions of o-cresol or p-cresol had a moderate effect only

The related mono-cyclic compounds benzol phenol and sodium salicylate had no PVR reducing effect similar to that of the cresols

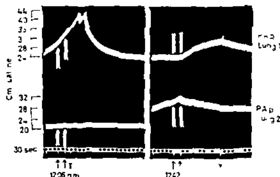


Fig 5 *Effects of cross-perfusion between two isolated lungs at times when one of the preparations showed an increase in PVR*

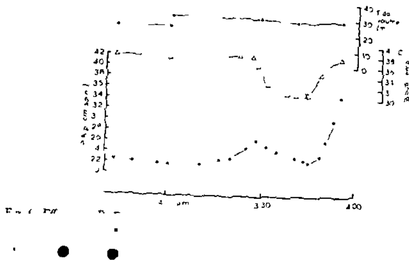
IPL's, lung 1 from rabbit, ♂, 2 9 kg lung 2 from rabbit ♀, 2 9 kg Perfusion of lung 1 started at 11 14 a m , of lung 2 at 11 42 a m BF for lung 1 150 ml/min, for lung 2 175 ml/min LAp 4 cm H O in both lungs Abbreviations as in Fig 2 The two lungs were perfused independently with blood from the same original pool Between arrows the outflowing blood from each lung was diverted into the blood reservoir of the other lung At T's 2 mg of tri-cresol injected into pulmonary arterial tubing of lung 1

Addition of tri-cresol in doses of from 0 5 to 6 mg did not alter the pulmonary vasoconstrictor responses to various agents such as bradykinin, kallidin histamine and 5 hydroxytryptamine Tri-cresol was thus not causing unspecific smooth muscle paralysis

The vasoconstrictor responses to noradrenaline were, however, somewhat reduced after addition to the perfusate of tri-cresol in the usual dose of 2 mg Larger doses would further reduce, and finally abolish, the constrictor response to noradrenaline a small vasodilator effect then becoming apparent Some recovery of the constrictor responses to noradrenaline was seen on further perfusion after addition of 1—2 mg of tri-cresol The ability of adrenaline to cause vasodilatation in the preparation was apparently unchanged after tri cresol addition

Tri-cresol addition did not cause any apparent changes in the viability or state of the preparation No tendency towards edema formation and no change in ability to oxygenate the blood was observed Weight changes and changes in tidal air went on as before the addition (Fig 4) In some experiments a slow and more moderate increase in PVR developed after the tri-cresol induced reversal of the initial rise in PVR This second, slow increase in PVR was not influenced by further tri-cresol addition, and it seemed to be most marked in experiments where the initial number of thrombocytes was high The general impression was however that tri-cresol addition resulted in a good and stable preparation which was well suited for various types of tests

Cross perfusion experiments In order to see if the progressive rise in PVR was due to a blood born substance of some stability cross-perfusion experiments were carried out Two lungs were perfused separately with blood from the same original pool one perfusion being started about half an hour before the other When the increase in PVR occurred in the first of these preparations lung 1 its outflowing blood was led over into the reservoir of the other preparation (lung 2) Simultaneously the blood from lung 2 was led into the reservoir of lung 1 The exchange of blood was carried out for a period long enough to allow the major part of the perfusate to be changed In the experiment of Fig 5 it will be seen that the change of blood did not influence the PAp-level in lung 2, nor did it reduce the markedly increasing PVR in lung 1 Shortly after the end of this cross-perfusion period, 2 mg of tri-cresol were injected into the pulmonary



arterial tubing of lung 1, whereupon the increasing PAp started to fall A second period of cross perfusion was started 36 min later, during a period of increasing PVR in lung 2 This time the exchange of blood between the preparations led to a marked increase in PAp in preparation no 1 whereas the PAp began to fall in preparation no 2

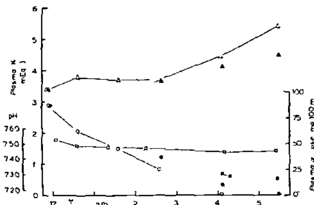
These puzzling results which were confirmed in another similar experiment show that under the conditions of the first blood exchange the tendency towards an increasing PVR could not be mediated via the perfusate from one preparation to the other Under the conditions of the second blood exchange, however, such a mediation occurred

Effect of temperature changes upon the increase in PVR The gradual unavoidable increase in PVR at blood temperatures around 37° C could be delayed for up to two hr by perfusing the lungs at a blood temperature of 31—35° C Once the increase appeared at the lower temperatures it seemed to develop rather slowly

The increase in resistance in preparations perfused at 37° C could also be temporarily halted by lowering the perfusate temperature (Fig 6) A reduction in blood temperature of some 3 C could change an increasing PVR to a falling one When the blood temperature was subsequently increased a new and marked rise in PVR occurred often after a rise in blood temperature of as little as 0 5—1° C Repeated periods with reductions and subsequent increases in perfusate temperature exhausted the vasoconstrictor tendency in some preparations so that on further perfusion at 37 C the PAp could be maintained at a low and stable level

Changes in the perfusate with time of perfusion The plasma glucose concentration the level of plasma K the blood pH the degree of hemolysis and the hematocrit were followed throughout the perfusion period in several experiments Fig 7 shows the development of 3 of these parameters during a 6 hr perfusion period with a 4 % CO_2 in air ventilation unbroken curves The same parameters were also followed in another portion of the perfusate blood which was incubated in an 4 % CO_2 in air atmosphere at 37 C for the same length of time

Plasma glucose concentration usually fell from some 100 mg/100 ml at the outset of the perfusion to unmeasurably low concentrations after 3—4 hr No correlation was



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found between plasma glucose levels and the time of onset of the increase in PVR Nor did addition of glucose to give a normal blood concentration prevent or influence the increase in PVR

Plasma K^+ usually rose throughout the perfusion the total rise for a 5 hr period being in the order of 2 meq/l

Perfusate pH during the first hr of perfusion in 29 typical experiments ranged from 7 32 to 7 55 with a mean value of 7 42 and with concentration of the values around the mean A gradual fall in perfusate pH of some 0 02 units/hr was usually seen in preparations continuously ventilated with 4 % CO_2 in air

Hemolysis of about the same degree occurred in all experiments where the perfusate was examined The most marked increase in hemolysis was seen when the pump and the perfusion circuit were being filled and before the start of lung perfusion Within the first hr of perfusion the concentration of hemoglobin in plasma reached about 0 1 g 100 ml increasing to about 0 25 g/100 ml after 4 hr of perfusion In a control portion of blood incubated at 37 C in an atmosphere of 4 % CO_2 in air hemolysis values were about one half of those seen in the perfusate

No correlation was seen between perfusate pH plasma K level or degree of hemolysis on the one side and the time of onset or degree of the PVR rise on the other

Hematocrit of the perfusate showed a moderate but steady increase Where injections and infusions were not carried out and where pulmonary edema was not induced the increase in hematocrit was in the order of 1 5—2 per cent in the course of a 5—6 hr experiment

Effects of ADP and ATP ATP has been reported to cause pronounced vasoconstriction in the pulmonary vascular bed of cats Emmelin and Feldberg 1948 The effect of injected or infused ATP and ADP was also evaluated in our preparation Small doses of both substances caused vasodilatation especially at the outset of the perfusion

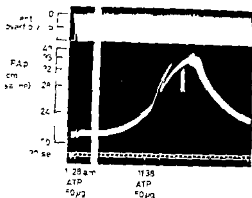


Fig 8 *Change in vasoconstrictor effect of a standard dose of ATP injected into pulmonary arterial tubing in a period of increasing PVR*

IPL, rabbit, ♀, 3 1 kg The marked constrictor response of the second injection of ATP [illegible]

a m LAp 4 cm H_2O Abbreviations as in Fig 2

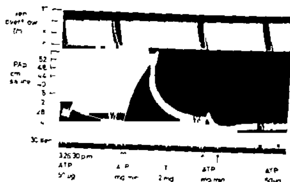


Fig 9 *Effects of injections and infusions of standard doses of ATP in the pulmonary arterial tubing before and after an addition to the perfusate of 2 mg of tri-cresol (T)*

IPL rabbit ♀, 2 9 kg Infusions of ATP carried out between arrows Tri-cresol was added at T Perfusion started at 11.26 a m BF 144 ml/min LAp 4 cm H_2O TV decreased from 49 to 48 ml during the period illustrated Abbreviations as in Fig 2

Both substances caused pulmonary vasoconstriction, however, when injected or infused in doses above a certain level Usually 20 µg of ATP would cause a significant rise in PAp ADP was 2—5 times more potent than ATP in our preparation On injection of ADP a fall in the number of circulating thrombocytes occurred, as would be expected with the ability of ADP to cause thrombocyte aggregation (Gaarder *et al* 1961) This could however not be the main reason for increased PVR on ADP injections or additions as the effect of ADP as well as that of ATP could be obtained in preparations with less than 10 000 thrombocytes mm^3 in the blood perfusate and also when cell free plasma was used as perfusate

During the early stage of perfusion the vasoconstrictor response to ATP or ADP progressively increased the response being especially marked after the gradual rise in PVR had started In the experiment of Fig 8 where the PVR increase was relatively sudden and rapid a dramatic increase in the response to 50 µg of ATP occurred within 10 min In other experiments where PVR increased more slowly the responses to ATP also changed much more gradually The vasoconstrictor response to ATP was markedly reduced when the perfusate temperature was reduced by 5—6 Addition of 2 mg of tricresol to the prefusate also reduced the response to standard doses of ATP (Fig 9) and ADP very markedly This interference of tri-cresol with ATP-ADP responses was somewhat transient the responses increasing moderately again with time Full recovery was never seen during perfusion periods of up to 3 hr Further addition of tri-cresol to the perfusate would again markedly reduce or almost abolish the vasocon-

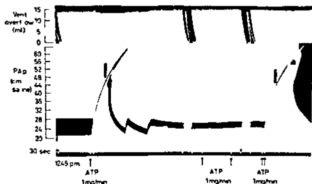


Fig 10 [illegible]

strictor responses to certain doses of ATP and ADP A complete inhibition of the vasoconstrictor effects of ATP and ADP was not obtained with tri-cresol in doses up to 6 mg Sufficiently large doses of ADP and ATP could always cause vasoconstriction in the preparation afterwards

The effect of infusions of ATP into the pulmonary arterial tubing did also change during the first part of the perfusion Small infusion doses which early in the perfusion caused a fall in PVR could later cause a marked and progressive increase in PVR (Fig 9, 1st infusion) After the addition to the perfusate of 2 mg of tri-cresol the marked vasoconstrictor response to an infusion of ATP into the arterial tubing was drastically reduced (Fig 9, 2nd infusion)

ATP is rapidly inactivated in blood In accordance with this, Fig 10 demonstrates that an infusion of ATP into the blood reservoir caused no vasoconstrictor response, whereas infusions of ATP in the same dose and at the same rate into the pulmonary arterial tubing gave drastic vasoconstrictor responses

Discussion

The increase in PVR occurring in isolated blood perfused rabbit lungs is an interesting problem and a serious obstacle to the full use of this preparation A mechanical obstruction of the vascular bed through trapping of thrombocytes or a liberation of 5-hydroxytryptamine or histamine from thrombocytes was apparently not causing the increase in PVR, since the phenomenon occurred also in preparations where nearly all thrombocytes had been removed from the perfusate Furthermore, cooling of the perfusate blood, which is known to augment platelet adhesiveness (Hellem 1960 resulted in a fall in PVR in our preparation

Histamine and 5-hydroxytryptamine might be liberated into the perfusate from sources other than the thrombocytes A high blood concentration of these two vasoactive substances could, however not be the mechanism giving the increase in PVR, since this increase was unaffected by the addition of the inhibitors Anthisan® and Deseril®

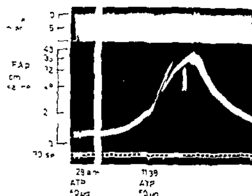


Fig 8 *Change in vasoconstrictor effect of a standard dose of ATP* . . .

. . . ATP necessitated a flow reduction from 300 to 250 ml/min by pump adjustment Arrow addition to the blood reservoir of 2 mg of tri-cresol (T) Perfusion started at 11 08 a m LAp 4 cm H_2O Abbreviations as in Fig 2

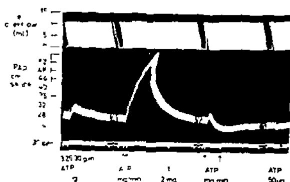


Fig 9 *Effects of injections and infusions of standard doses of ATP into the pulmonary arterial tubing before and after an addition to the perfusate of 2 mg of tri-cresol (T)*

IPL, rabbit ♀ 2 9 kg Infusions of ATP carried out between arrows Tri-cresol was added at T Perfusion started at 11.5 a m BF 144 ml min LAp 4 cm H_2O TV decreased from 49 to 48 ml during the period illustrated Abbreviations as in Fig 2

Both substances caused pulmonary vasoconstriction however, when injected or infused in doses above a certain level Usually 20 μg of ATP would cause a significant rise in PAp ADP was 2—5 times more potent than ATP in our preparation On injection of ADP a fall in the number of circulating thrombocytes occurred, as would be expected with the ability of ADP to cause thrombocyte aggregation (Gaarder *et al* 1961) This could however not be the main reason for increased PVR on ADP injections or additions as the effect of ADP as well as that of ATP could be obtained in preparations with less than 10 000 thrombocytes mm³ in the blood perfusate and also when cell free plasma was used as perfusate

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was somewhat transient the response . . .
recovery was never seen during . . .
tri-cresol to the perfusate would again markedly reduce or almost abolish the vasocon-

The possible role of microorganisms for the changes in the preparation is difficult to asess We have found it difficult to prevent the growth in the blood of microbes which are resistant towards penicillin and streptomycin A direct and immediate effect of microbes as the cause of the time-dependent increase in PVR seems unlikely, as the tendency towards PVR increase could not be transferred via the blood from an older to a younger preparation Microbes could, however, play a role in some gradual change of the preparation

The effect of tri-cresol on the increasing PVR in the isolated rabbit lungs is a very interesting one Whatever the mechanism behind this effect, tri-cresol has become a very useful agent in enabling better use of the preparation

The cresol effect seems to be a fairly specific one, mainly affecting the responses to ATP and ADP, to some extent also the response to noradrenaline The effect is caused by as little as 4—8 *μg/ml of perfusate Two of the three isomers (o-cresol and p-cresol)* are much more active than the third one, m-cresol The related compounds benzol phenol and sodium salicylate are without effect Further studies on the effect of cresols in isolated lungs as well as in other organs seems indicated Cresols are normally found as intermediates in the deamination of amino acids in the intestine They also occur in urine Their interference with effects caused by ATP and ADP might be of general interest and have wider aspects than those releated to the special situation in our preparations

We want to thank A Lystad Kapt W Wilhelmsen og Frues Bakteriologiske Institutt University of [illegible]
M [illegible]
A [illegible]
Hu [illegible]
Fabriks Medicinske fond from Anders Jahres Fund for the Promotion of Science from Eli Lilly and Company Indianapolis U S A from the Nansen Foundation and from the Norwegian Council on Cardiovascular Diseases is gratefully acknowledged

References

ALDRIDGE W N and H B STONER The behaviour of liver mitochondria isolated from rats with different body temperature after limb ischaemia or after injection of 3 5-Dinitro-o-cresol *Biochem J* 1960 *74* 148—154

ALLISON P R I de BURGH DALY and B A WAALER Bronchial circulation and pulmonary vasomotor nerve responses in isolated perfused lungs *J Physiol* (Lond 1961 *157* 462—474

BORN G V R Acute oedema in the isolated perfused lungs of rabbits *J Physiol* Lond 1954 *124* 502—514

BRECHER G and E P CRONKITE Morphology and enumeration of human blood platelets *J appl Physiol* 1950 *3* 365—377

DALY I DE B and B A WAALER The effect of bronchial vascular system perfusion on the pulmonary vascular resistance (PVR in isolated lung lobes of the dog *Quart J exp Physiol* 1961 *46* 272—282

DRABKIN D L [illegible] itions from washed [illegible] 1935 *112* 51—65

EM [illegible] *Brit J Pharmacol* [illegible]

ENGELBERG J and A B DUBOIS Mechanics of pulmonary circulation in isolated rabbit lungs *Amer J Physiol* 1959 *196* 401 414

EULER U S von and F LISHAJKO Dopamine in mammalian lung and spleen *Acta physiol pharmacol neerl* 1957 *6* 295 303

GAARDER A J JONSON S LALAND A HELLEM and P A OWREN Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature* Lond 1961 *192* 531—532

HAMBERG, U and M ROCHA E SILVA, On the release of bradykinin by trypsin and snake venoms *Arch int Pharmacodyn* 1957 *110* 222—238

HALGE, A, P K M LUNDE and B A WAALER The effect of bradykinin kallidin and eledoisin upon the pulmonary vascular bed of an isolated blood perfused rabbit lung preparation. *Acta physiol scand* 1966 *66* In press

HELLEM, A. J, The adhesiveness of human blood platelets in vitro *Scand J clin Lab Invest* 1960 *12* Suppl 51

HUGGETT, A S G and D A NIXON Use of glucose oxidase, peroxidase and o-dianisidine in determination of blood and urinary glucose. *Lancet* 1957 *II* 368—370

HUGHES F B and B B BRODIE, The mechanism of serotonin and catecholamine uptake by platelets *J Pharmacol exp Ther* 1959 *127* 96—102

[illegible]

[illegible]

protein fractions *Amer J Physiol* 1964 *206* (4) 923—925

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Effect of Exercise on Plasma Potassium in Man

By

HELGE LAURELL and BENGT PERNOW

Exercise leads to an increase in the blood plasma concentration of potassium, which has been shown both in man (Laurell and Pernow 1961, Hultman and Bergstrom 1962) and in dogs (Kjellmer 1965) From this finding, as well as the similarity in vascular response to exercise and potassium infusion, Kjellmer (1965) suggested that the hyperemia of exercise was due to the local release of potassium

The changes in potassium concentration of arterial and femoral venous plasma during and after severe leg exercise (1 200—1 800 kpm per min) on a bicycle ergometer was studied in 6 healthy male subjects In 3 other subjects blood was simultaneously drawn from the brachial artery, a deep and a superficial vein of the forearm during and after exercise with a hand ergometer The blood samples analyzed for potassium oxygen saturation, lactate, pH and hematocrit, were obtained from teflon catheters with heparinized glass syringes Plasma showing hemolysis was discarded Potassium was determined by an Eppendorf flame photometer an no correction was used for protein For further details of the procedure and methods see Carlson and Pernow (1961)

At rest a negative arterio-venous (AV) potassium concentration was observed in most cases During exercise there was a considerable increase in both arterial and venous concentration of potassium with a more accentuated negative AV difference After exercise the concentration of potassium fell rapidly and, 30 sec after the end of work, had already decreased to or even below the basal level In all cases a positive AV difference was obtained during [illegible]
test the arterio-deep venous concer [illegible]
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potassium predominatingly in the muscles

The increase in serum potassium during exercise was well correlated to the increase in heart rate and lactate as well as to the decrease in venous oxygen saturation and pH (Fig 1) After exercise however the plasma concentration of potassium changed much more rapidly than the other parameters Hematocrit increased during exercise 5—8 per cent

This investigation was supported by grants from Karolinska Institutet (Stiftelsen Gustaf och Tyra Svenssons Minne and the Swedish Medical Research Council.

TABLE I Plasma concentration of potassium (µmoles/ml) at rest during and after exercise on a bicycle ergometer or with a hand ergometer The values given for leg exercise were those obtained at the highest load (1 500—1,800 kpm/min) FV = femoral vein DV = deep forearm vein A = artery

	Rest		Exercise		After exercise					
					30 sec		5 min		20 min	
	FV	A V	FV	A V	FV	A V	FV	A V	FV	A V
Leg exercise (6 cases)										
Mean	4.61	0.08	6.15	0.24	5.19	0.21	3.91	0.42	4.9	0.0[illegible]
±1 SEM	0.14	0.045	0.14	0.06	0.31	0.14	0.09	0.27	0.10	0.06
Forearm exercise (3 cases)										
	DV	A V	DV	A V	DV	A V	DV	A V	DV	A V
Mean	4.70	0.03	5.68	0.62	4.82	0.23	4.35	0.45	4.73	0.12

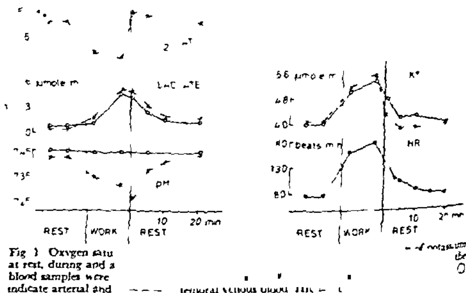


Fig 1 Oxygen satu[illegible] at rest, during and a[illegible] blood samples were [illegible] indicate arterial and — — — femoral venous blood [illegible] of potassium [illegible]

References

CARLSON L. A. and B PERNOW Studies on the peripheral circulation and metabolism in man. I Oxygen utilization and lactate pyruvate formation in the legs at rest and during exercise in healthy subjects *Acta physiol scand* 1961 52 328—342

HULTMAN E. and J BERGSTRÖM Plasma potassium determination. *Scand J clin Lab Invest.* 1962 64 87—93

KJELLMER I The potassium ion as a vasodilator during muscular exercise *Acta physiol scand* 1965 63 450 -468

LAURELL, H and B PERNOW *Arterio-venous plasma potassium differences during exercise (in Swedish) Abstr Com Swed Med Ass Meet* 1961

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From the Istituto Superiore di Sanità, Rome, Italy, and the Department of Histology, Karolinska institutet, Stockholm, Sweden

Catecholamine Containing Neurons in the Cockroach Brain

By

NORA FRONTALI and KARL-AXEL NORBERG

Insects have been shown to contain high concentrations of catecholamines, particularly dopamine (Östlund 1954), while noradrenaline and adrenaline occur in lower concentrations (Euler 1961) However the cellular localization has not been known

The present paper reports the histochemical demonstration of catecholamines in the brain of *Periplaneta americana* About 50 brains of adult cockroaches (both sexes) were dissected out and treated for fluorescence microscopy according to the formaldehyde method of Falck and Hillarp (for references see Norberg and Hamberger 1964, Falck and Owman 1965)

Prominent groups of nerve cells exhibiting the yellow-green fluorescence characteristic of catecholamines are found in various parts of the brain In the protocerebrum[1] they occur either isolated or in small groups located close to the surface of the protocerebral lobes, near the outer border of the β lobe (Fig 1 and 2), dorsally in the pars intercerebralis, and just under the calyces The optic lobe contains small, faintly fluorescent, scattered cells located near the surface In the deutocerebrum groups of large, strongly fluorescent cells are found outside the antennal glomeruli Also the tritocerebrum and the subesophageal ganglion (Fig 3) contain single fluorescent cells Varicose terminals with the same type of yellow-green fluorescence as exhibited by the cell bodies are found to form dense networks in the protocerebral lobes (Fig 2 and 4) around the central body, and enclosing the antennal glomeruli (Fig 2) The α lobe (Fig 2), and especially the β-lobe (Fig 1 and 2) exhibit intense specific fluorescence with striking cross-striations and prominent differences in fluorescence intensity among the various parts Also the central body and a narrow striation in one of the optic ganglia show strong fluorescence of the same type In the four latter structures the fluorescence appears to have an amorphous distribution but it seems probable that it is localized in nerve terminals which are not visible under the present conditions In fact recent electron microscopical work on the β lobe supports this view (Crateri and Frontali, in preparation)

[1] Nomenclature according to Horridge (1965)



Fig 1 β-lobe brain of *Periplaneta americana* Note the strongly fluorescent "cross striation". Fluorescence apparently amorphous, probably located in submicroscopical nerve terminals × 130

Fig 2 Bra n Per... to the left ... Right anc ... cell bodie ... networks of fluorescent synaptic nerve terminals arranged in "baskets" around the antennal glomeruli × 50

Fig 3 Subesophageal ganglion, *Periplaneta americana* A single strongly fluorescent cell with a non fluorescent nucleus and a faintly fluorescent axon × 19[illegible]

Fig 4 Protocerebral lobe, brain of *Periplaneta americana* Dense networks of intensely fluorescent varicose nerve terminals × 200

The present results thus give evidence for the existence of catecholamine-containing neurons in the cockroach brain, and give strong morphological support to the view that catecholamines act as transmitters there

This investigation has been partly supported by a research grant (B66—257) from the Swedish Medical Research Council

References

CORRODI H N-A HILLARP and G JONSSON, Fluorescence methods for the histochemical demonstration of monoamines 3 Sodium borohydride reduction of the fluorescent compounds as a specificity test *J Histochem Cytochem* 1964 *12* 582 586

EULER U S VON Occurrence of catecholamines in acrania and invertebrates *Nature* (Lond) 1961 *190* 170—171

FALCK, B and CH OWMAN A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic amines *Acta Univ Lund* 1965 Sect II no 7 1—23

HORRIDGE G A Arthropods general anatomy In *Structure and function of the nervous system of invertebrates* by T. H. Bullock and G A Horridge W H Freeman and Co 1965

NORBERG, K -A and B HAMBERGER, The sympathetic adrenergic neuron Some characteristics revealed by histochemical studies on the intraneuronal distribution of the transmitter *Acta physiol scand* 1964 *63* Suppl *239* 1 42

ÖSTLUND, E. The distribution of catecholamines in lower animals and their effect on the heart *Acta physiol scand* 1954 *31* Suppl 112

Acta physiol scand 1966 66 245—246

From the Department of Histology, Karolinska Institutet Stockholm Sweden

Further Evidence for the Existence of Tubero-infundibular Dopamine Neurons

By

Kjell Fuxe and Tomas Hokfelt

Previous studies (Fuxe 1964) have given strong support for the existence of a tubero-infundibular dopamine (DA) neuron system Further evidence for this view is given in the present paper, using the histochemical fluorescence method for the cellular demonstration of catecholamines (CA) and 5 hydroxytryptamine (Falck *et al*) in combination with an experimental pharmacological approach

About 25 adult Sprague Dawley rats were used Two types of experiments were made (1) Hypophysectomy was performed on 15 rats via the parapharyngeal or transauricular approach in order to damage the median eminence The animals were sacrificed 1, 2 and 3 days after the operation (2) 10 rats were treated with reserpine (10 mg/kg i p, 24 hrs before killing) — nialamide (100 mg/kg—300 mg/kg i p, 5 hrs before killing) — 3 4-dihydroxy phenylalanine (l dopa) (50 mg/kg—100 mg/kg s c, 30 min before killing) in order to increase the amine contents of the central CA neurons — All the animals were killed by decapitation after light chloroform anesthesia The hypothalamus with the infundibular stalk was dissected out freeze-dried treated with formaldehyde gas embedded sectioned and mounted as previously described in detail (Dahlstrom and Fuxe 1964)

The operated animals that had an intact median eminence showed a normal fluorescence microscopical picture with only a small number of weakly green fluorescent CA nerve cells in the anterior part of the nucleus arcuatus (Fig 1) and the ventral part of the anterior periventricular nucleus (group A12 see Dahlstrom and Fuxe 1964) The DA terminals in the external layer of the median eminence of these animals formed a strongly fluorescent zone of a dotted appearance In the operated animals that had a damaged median eminence however, a marked increase was observed in the number and intensity of the CA nerve cells of group A12 (Fig 2) The increase was related to the size of the lesion *i.e* an extensive damage caused a strong increase in intensity and number of cellbodies The most pronounced changes were observed one day after operation These changes in the amine contents of the CA cell bodies in all probability represent retrograde cell body changes (*cf* Dahlstrom and Fuxe 1965

In those animals that had been treated with reserpine nialamide l-dopa it was possible in some cases to trace weakly green fluorescent axons from the cell bodies of





Fig 1 Nuc arcuatus of normal rat A small number of weakly green fluorescent CA nerve cells (→) are observed E = ependyma of the third ventricle × 270

Fig 2 Nuc arcuatus of a rat with a damaged median eminence The operation was made 1 day before killing A large number of CA nerve cells with an increased fluorescence intensity are present E ependyma of the third ventricle × 270

the nuc arcuatus to the DA terminals of the median eminence (see also Fuxe 1963 The A12 nerve cells showed a very marked increase in number and fluorescence intensity as did the DA terminals of the median eminence These findings are supported by other workers (Lichtensteiger and Langemann 1965)

The present study together with previous studies (Fuxe 1964) give final evidence for the existence of tubero-infundibular DA neurons Their function is at present under study

The skilful technical assistance of miss Vivian Bring is gratefully acknowledged
This work has been supported by the Swedish Medical Research Council (Project No 12 715 01 and by a Public Health Service Research Grant (NB 05236-01)

References

DAHLSTRÖM A and K FUXE Evidence for the existence of monoamine-containing neurons in the central nervous system I Demonstration of monoamines in the cell bodies of brain stem neurons Acta physiol scand 1964a 62 Suppl 1—55

DAHLSTRÖM A and K FUXE Evidence for the existence of monoamine neurons in the central nervous system II Experimentally induced changes in the intraneuronal amine levels of bulbospinal neuron systems Acta physiol scand 1965 64 Suppl 247

FALCK B N A HILLARP G THIEME and A TORP Fluorescence of catecholamines and related compounds condensed with formaldehyde J Histochem Cytochem 1962 10 348—354

FUXE K Cellular localization of monoamines in the median eminence and the infundibular stem of some mammals Z Zellforsch 1964 61 710—724

FUXE K The distribution of monoamine nerve terminals in the central nervous system Acta physiol scand 64 Suppl 247

LICHTENSTEIGER W and H LANGEMANN Aufnahme exogener Katecholamine in monoamin-haltige Neurone des ZNS Helv physiol pharmacol Acta 1965 23 c 31 c33

Acta physiol. scand. 1966. 66. 247—248

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Pyruvate, Glutamate and Tricarboxylic Acid Intermediates in the Crustacean Stretch Receptor Neurone after Prolonged Impulse Activity

By

Ezio Giacobini and Pier Carlo Marchisio

Giacobini and Marchisio (1965) studied the action of 7 intermediates of the tricarboxylic acid cycle (TCA), and also pyruvate and glutamate on the impulse activity and oxygen uptake of the slowly adapting stretch receptor neurone (SRN)

It was felt that the physiological significance of the different steps of the TCA cycle in connection with the impulse activity of this neurone could be better analyzed by determining the subsequent variations of the substrate levels after physiological stimulation

The SRN used in this investigation were dissected from abdominal segments of crayfish (*Orconectes virilis*) The technique we have been following for handling the preparation through the various steps is basically the same as described by Giacobini and Grasso (1965) The analytical methods used were based on the fluorimetric measurement of pyridine nucleotides in the [illegible] analysis (Bergmeyer, 1963) All enzymes used were from Boehringer & Söhne The other reagents used in this investigation were of analytical grade

The measurements were carried out on 120 frozen-dried cell preparations at rest and after 5 and 10 10^4 impulses respectively

In this investigation the variation $\pm$ S.E.M of the following 4 substrates will be reported pyruvate, isocitrate, glutamate and α ketoglutarate

The cellular content of pyruvate decreases from 432 $\pm$ 210 μmoles / kg w w at rest to 295 $\pm$ 147 μmoles / kg w w after 5 10^4 impulses, and to 210 $\pm$ 58 μmoles / kg w w after 10 10^4 impulses In terms of resting values, these variations represent a 51 % decrease which was found to be highly significant (Fig 1

This decrease of pyruvate may account for the increase of lactate demonstrated by Giacobini and Grasso (1965) after a corresponding number of impulses The isocitrate concentration at rest is 59 $\pm$ 4 μmoles kg w w From this value the concentration falls after 5 10^4 impulses to 51 $\pm$ 1 μmoles / kg w w, and after 10 10^4 impulses to 35 $\pm$ 5 μmoles / kg w w This variation represents a significant decrease of 41 % (Fig 1) The isocitrate change is somewhat difficult to interpret before an overall view of the TCA cycle is achieved

Glutamate was found to be 379 $\pm$ 22 μmoles / kg w w in resting cells. Upon stimulation it increased to 462 $\pm$ 40 μmoles kg w w after 5 10^4 impulses, and to 578 $\pm$ 129 μmoles / kg w w after 10 10^4 impulses, a percentage increase of 52 % (Fig 1

After the same number of impulses the α-ketoglutarate concentrations did not vary significantly (Fig 1) The levels were 51 $\pm$ 6 μmoles / kg w w at rest 53 $\pm$ 22 μmoles / kg w w and 53 $\pm$ 9 μmoles kg w w after 5 10^4 and 10 10^4 impulses respectively

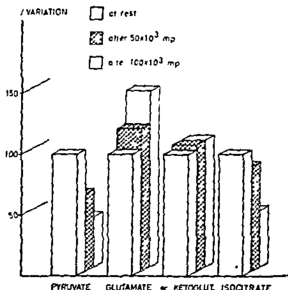


Fig 1 Percentage changes in four intermediates of the slowly adapting stretch receptor cell after 50 10³ and 100 10³ impulses The figure is based on the average values reported in the text

Compared to other preparations of nerve tissue, the glutamate content of the crustacean nerve cells is lower than in crustacean fibers (Cheng and Waelsch 1963) and in mammalian nerve tissue (Waelsch *et al* 1964) The cell body and the axon contributes in practically equal parts to the weight of this preparation which suggests a higher concentration of glutamate in the axon This finding may have some implication on the metabolism and function of GABA in the axonal structures As the concentration of α ketoglutarate the intermediate percursor of glutamate did not show any variation after prolonged activity it may indicate a continuous replenishement by the TCA cycle

Our results indicate that in the crayfish receptor neurone, as in frog nerve, pyruvate is oxidized to yield energy for maintenance of excitability

This investigation has been supported by grants from the U S Public Health Service National Institute of Health NB 04561-01 04561-02 and 04561-03 and by grants from the Swedish Medical Research Council project No U380 W101 and 12 X 246-01

References

Bergmeyer H U *Methods of enzymatic analysis* Verlag Chemie (Weinheim) and Academic Press New York and London 1963 pp 384—388 and 318 323

Cheng S C and H Waelsch Some quantitative aspects of the fixation of carbondioxide by the lobster nerve *Biochem Z* 1963 338 643 –653

Giacobini E and A Grasso Variation of glycolytic intermediates phosphate compounds and pyridine nucleotides after prolonged stimulation in an isolated crustacean neurone *Acta physiol scand* 1966 66 49 57

Giacobini E and P C Marchisio The action of tricarboxylic acid cycle intermediates and glutamate on the impulse activity and respiration of the crayfish stretch receptor neurone *Acta physiol scand* 1966 66 58 66

Lowry O H J V Passonneau D W Schulz and M K Rock The measurement of pyridine nucleotides by enzymatic cycling *J biol Chem* 1961 236 2746—2755

Lowry O H J V Passonneau F X Hasselberger and D W Schulz Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain *J biol Chem* 1964 239 18—30

Lowry, O H and J V Passonneau 1965 Pers comm

Waelsch H S Berl C A Rossi D D Clarke and D P Purpura Quantitative aspects of CO_2 fixation in mammalian brain in vivo *J Neurochem* 1964 11 717 728

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Effect of Posterior Pituitary Hormones on the Myometrial Response to Prostaglandin

By

Rune Eliasson

In a study by Eliasson and Posse (1960) on 8 healthy fertile young women it was observed that the intravaginal application of a total prostaglandin extract from human seminal fluid (HSF PG) caused an increase in the motility of the non pregnant human uterus in situ at the estimated time of ovulation. In two of the subjects the increased motility was followed after 20—25 min by a period of markedly decreased activity. It was postulated that this response could be due to a continuous absorption of the active compounds thereby increasing the blood (or tissue) concentration of prostaglandins. The reaction would thus be similar to that observed in vitro, i.e. a small dose of HSF PG or prostaglandin E_1 (PGE_1) causing a stimulatory effect, while a slightly larger dose causes the usual inhibitory effect (Eliasson 1959, Bygdeman and Eliasson 1963a, Bygdeman 1964).

According to Csapo (1954) oxytocin exerts its stimulatory effect by sensitizing the myometrium to various intrinsic and extrinsic stimuli. It was therefore thought that infusion of oxytocin might increase the reactivity of the myometrium also to prostaglandin thus evoking the inhibitory response more rapidly. Oxytocin has no effect on the motility of the non pregnant human uterus in vitro or in vivo, while vasopressin is a potent stimulator (Eliasson and Posse 1963, Bygdeman and Eliasson 1963b). It is likely that, in man, oxytocin and vasopressin are released from the posterior pituitary gland simultaneously in connection with sexual stimulation (for ref. see Pickford 1960). For these reasons, a combination of the two hormones (Hypadrin® ASTRA) was used.

During i.v. infusion of 100—200 mU of each of vasopressin and oxytocin per minute in two women at the estimated time of ovulation, intravaginal application of HSF PG caused a rapid and marked decrease in the activity without the initial period of increased activity. During the proliferative phase prostaglandin usually had no effect, but in one subject receiving an i.v. infusion of vasopressin and oxytocin (120 mU of each per min) there was a marked increase in the uterine activity following the intravaginal application of HSF PG. The results indicate that in man simultaneous infusion of oxytocin and vasopressin may increase the sensitivity of the myometrium in a way similar to that described for oxytocin in rabbits (Csapo 1954).

In order to further evaluate this postulated effect, a number of in vitro experiments have been performed. Strips (2 × 2 × 20 mm) were taken from nonpregnant uteri after hysterectomy and suspended in an isolated organ bath containing a slightly modified Tyrode solution aerated with 5 % CO_2 in oxygen (pH 7.4 ± 0.05, 37° C). The movements were recorded on smoked drums with levers (amplification 1:25). The method has been fully described elsewhere (Bygdeman and Eliasson 1963a, Bygdeman 1964). The reported results are based on 16 strips from 6 uteri.

Lysin vasopressin and oxytocin (Syntocinon) was added alone or in combination either directly to the bath about 1 min before the addition of prostaglandin or to the perfusion fluid so as to give a continuous concentration during the whole experiment. The concentration of vasopressin or oxytocin was 0.025—0.3 mU/ml bath fluid. Prostaglandin E_1 was usually added in doses of 0.05—0.5 μg/ml in order to cause a moderate inhibition of the spontaneous motility. In none of the experiments the effect of prostaglandin E_1 on the myometrium was increased or decreased.

The reason for this difference between the in vivo and in vitro findings is not clear. If the potentiating effect of vasopressin in vivo can be confirmed in an extended study the possibility that vasopressin changes the response to prostaglandin by increasing the permeability of the vagina to the active compounds has to be considered. That vasopressin can influence the permeability of some other mucous membranes is well known (*cf.* Leaf and Hayes 1961, Sawyer 1961).

This investigation was supported by grants from the Swedish Medical Research Council (Project No. Y f
fully acknowled
E (PGE_1) was
and Syntocinon by Sandoz AG, Basel.

References

Bygdeman, M.: The effect of different prostaglandins on human myometrium in vitro. Acta physiol. scand. 1964. 63. Suppl. 242.
By ... man seminal fluid on the ... 1963. 59. 43—51.
By ... on the motility of
Csapo, A.: Dependence of isometric tension and isotonic shortening of uterine muscle on tem- ... 1951. 177. 348—354.
... ological action ...
... gnant human uterus
... on permeability and ... 7—49?
... Ed. M. Schacher
Pergamon Press, London 1960. 42—48.
Sawyer, W. H.: Neurohypophyseal hormones. *Pharmacol. Rev.* 1961. 13. 225—277.

Acta physiol. scand. 1966. 66. 251—252

From the Department of Anatomy University of Helsinki, Finland

Effect of Hydrocortisone on Histochemically Demonstrable Catecholamines of the Para-aortic Body of the Rat

By

MATTI LEMPINEN

The main para aortic body (the Organ of Zuckerkandl) of the rat degenerates soon after birth losing its chromaffin reaction Repeated injections of cortisone or hydrocortisone not only prevent the postnatal degeneration of the extra-adrenal chromaffin tissue but bring about an increase in its catecholamine content (Lempinen 1964)

In the present study, the effect of hydrocortisone treatment was studied, using in addition to the chromaffin reaction, two histochemical reactions specific to noradrenaline

Rats [illegible]

The main para-aortic body of a newborn rat exhibited a strong uniform chromaffin reaction (Fig 1) After calcium formalin fixation a brilliant green fluorescence was seen in the body (Fig 2) The iodate reaction was always negative

Hydrocortisone administration clearly increased the intensity of the chromaffin reaction (Fig 3), and no signs of cellular degeneration was observed On the other hand formalin induced fluorescence disappeared altogether (Fig 4) The iodate reaction remained negative, as in normal newborn rats

The results indicate that the extra adrenal chromaffin cells exhibit at birth formalin-induced fluorescence indicating the presence of noradrenaline, but do not give a positive iodate reaction Because the iodate reaction is less sensitive than the formalin induced fluorescence, this difference may be due to a low noradrenaline concentration in these cells Since hydrocortisone administration which obviously caused an increase in the total catecholamine concentration, as indicated by the chromaffin reaction, also caused disappearance of the formalin induced fluorescence, it seems that noradrenaline in hydrocortisone-treated animals is replaced by another amine or that it is stored in a less firmly bound form, diffusing away before formalin has immobilized it This question has been further studied in another report (Eränkö, Lempinen and Räisänen 1966)

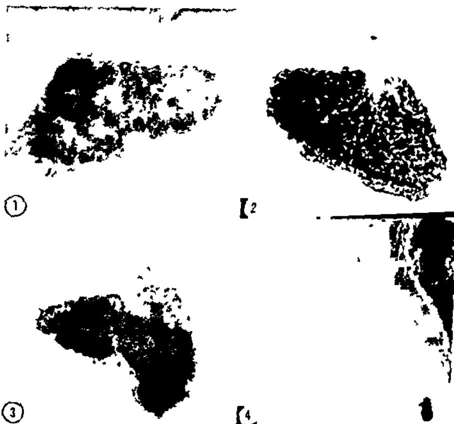


Fig 1 Main para aortic body of a newborn rat Chromaffin reaction.

Fig 2 Main para aortic body of a newborn rat Formalin induced fluorescence

Fig 3 Main para aortic body of a 9-day-old hydrocortisone treated rat Chromaffin reaction

Fig 4 Main para aortic body of a 9-day-old hydrocortisone treated rat Formalin induced fluorescence The whole body is essentially non fluorescent Magnification × 120

References

ERÄNKÖ O Histochemistry of noradrenaline in the adrenal medulla of rats and mice *Endocrinology* 1955 57 363 368

ERÄNKÖ O M LEMPINEN and L RÄISÄNEN Adrenaline and noradrenaline in the organ of Zuckerkandl and adrenals of newborn rats treated with hydrocortisone *Acta phys scand* 1966 66 253 254

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Acta physiol. scand. 1966. 66. 253—254

From the Department of Anatomy University of Helsinki, Siltavuorenpenger, Finland

Adrenaline and Noradrenaline in the Organ of Zuckerkandl and Adrenals of Newborn Rats Treated with Hydrocortisone

By

OLAVI ERÄNKÖ, MATTI LEMPINEN and LIISA RAISÄNEN

Adrenals of newborn rats contain noradrenaline (NA) and adrenaline (A) in the same proportion, about 1 5, as adrenals of adult rats (Eranko and Raisanen 1957) No such information is available of the extra-adrenal chromaffin tissue of the rat, although histochemical observations show that it contains catecholamines at birth but degenerates in about a week thereafter, unless adrenocortical hormone is given daily (Lempinen 1964) Treatment with hydrocortisone results in an increase in the intensity of the chromaffin reaction but in a loss of formalin-induced fluorescence (Lempinen 1965)

In the present study, the main para aortic chromaffin body, i.e the Organ of Zuckerkandl (OZ), and the adrenals were examined for NA and A in normal and hydrocortisone treated young rats

Newborn albino rats were injected daily with 0 1 mg of hydrocortisone for 7 days Since the extra adrenal chromaffin tissue has degenerated in normal 7-days-old rats, normal newborn rats were used as controls The animals were killed by decapitation and the retroperitoneal tissue block containing the OZ was frozen and sectioned serially The OZ and the adrenals were dissected from frozen dried sections Material thus collected from 6 animals was pooled and applied to the base line of the chromatography paper Small pieces of frozen dried adrenal medulla of adult rats served as controls The chromatogram was developed with phenol / 0 1 N hydrochloric acid and the catecholamines were made fluorescent by spraying with 2 % potassium ferricyanide in phosphate buffer at pH 7 1

A typical chromatogram is shown in Fig 1 The OZ of the newborn control rats (Z) contained NA only while both NA and A were found in the OZ of hydrocortisone-treated rats (Z'), the NA A proportion being about the same as that in the adult adrenal medulla (unmarked While hydrocortisone thus radically affected the catecholamine composition of the OZ it had apparently no such effect on the adrenal medulla, which contained both NA and A in newborn controls (A) and in hydrocortisone-treated (A') rats

The presented observations provide for the first time direct evidence of the effect of a cortical hormone on the methylation of catecholamines in chromaffin cells, which has been earlier suspected on a speculative basis (see review in Lempinen 1963 However, it is not

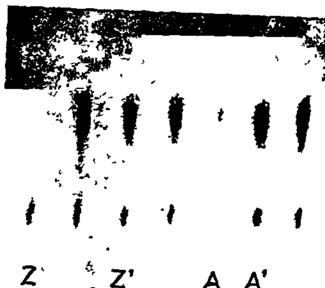


Fig. 1. Fluorescence ob [illegible] rats. The unmarked spots are obtained from adult adrenal medulla.

possible to say with certainty whether the appearance of A is directly due to hydrocortisone itself or to other factors which have the chance to become operative when the normal degeneration of the extra-adrenal chromaffin cells is prevented with the aid of hydrocortisone.

References

ERÄNKÖ, O. and L. RAISANEN: Adrenaline and noradrenaline in the adrenal medulla during postnatal development of the rat. *Endocrinology* 1957. *60*. 753—760.

LEMPINEN, M.: Extra-adrenal chromaffin tissue of the rat and the effect of cortical hormones on it. *Acta physiol. scand.* 1964. Suppl. *231*.

LEMPINEN, M.: Effect of hydrocortisone on histochemically demonstrable catecholamines of the para-aortic body of the rat. *Acta physiol. scand.* 1966. *66*. 251—252.

Acta physiol scand 1966 66

the Department of Physiology and the Department
Stockholm Sweden

Postnatal Changes in the Preaortal Pa
of Rabbits

By

Tomas Brundin, Bertil Hamberger and

In fetal and newborn rabbits the preaortal paragan
cholamines and presumably of physiological importan
(Brundin 1965) The postnatal fate of these organs
Pellegrini (1906) described degenerating paragangl
(e g Coupland 1956) have claimed that in this species
in the paraganglia

The present paper reports histochemical and other
from newborn young and adult rabbits The animals
or decap tation and the preaortal paraganglia were
tion of the catecholamines the organs were treated
condensation method of Falck and Hillarp (Falck 1
contents of catecholamines were determined accord
In newborn rabbits (Fig 1) the organ consisted of
yellow green fluorescence of high intensity In rabb
of the paraganglionic cells showed considerably lower
that these cells had partly lost their catecholamine con
connective and fat tissues occupied almost the ent
of fluorescent cells (Fig 2) The total catecholamine
increased slightly during the first postnatal week
decrease towards the adult stage

It is concluded that the preaortal paraganglia
involution Only small amounts of catecholamines

This study has been supported by grants from the
Fund and from the Swedish Medical Research Council



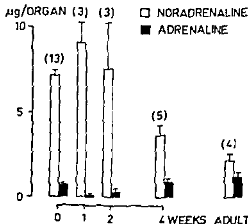
Fig 1



Fg 2

Fig 1 Paragangl on newborn rabbit The parenchymal cells show intense fluorescence due to a high catecholamine content × 110

Fig 2 Paraganglion rabbit two months old The largest part of the organ now cons sts of con nective and fat tissue and only small clusters of fluorescent cells rema n × 110



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References

BRUNDIN T Catecholamines n the preaortal paragangl a of fetal rabbits *A ta phy ol scand* 1965 *64* 287 288

COUPLAND R E The development and fate of the abdominal chromaff n tissue in the rabb t *J Anat (Lond* 1956 *90* 527 537

EULER U S v and F LISHAJKO Improved tech nique for the fluorimetric est mat n of cate cholamines *Acta physiol scand* 1961 *51* 348—355

FALCK B Observations n the poss b lities of the cellular local zati n of mo noam nes by a fluorescence method *Acta phy ol scand* 1962 *56* Suppl 197

FALCK B N A HILLARP G THIEME and A TORP Fluorescence of catecholam nes and related compounds condensed with formaldehyde *J Histochem Cytochem* 1962 *10* 348 354

PELLEGRINI E Contributo allo stud o dello m orphologia dell organ paras mpatico dello Zuckerkan ll *Monit ool ital* 1906 *17* 254—264

Acta physiol. scand. 1966. 66. 257—268

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The Influence of Thyroxine Treatment and Thyroidectomy on the Calorigenic and Some Other Metabolic Effects of Adrenaline and Noradrenaline in Experiments on Fasted Rabbits

By

NILS SVEDMYR

Received 27 April 1965

Abstract

Svedmyr, N. *The influence of thyroxine treatment and thyroidectomy on the calorigenic and some other* met ... and
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The relationship between the physiological and pharmacological effects of the catecholamines (CA) and of the thyroid hormones has been discussed in reviews by Ellis (1956), Hoch (1962), Harrison (1964) Rosenberg and Bastomsky (1965), Lundholm, Mohme Lundholm and Svedmyr (1966)

Among the reactions which have attracted special attention are the mutual influence of thyroid hormones and CA on their calorigenic effects

In the following the influence of the thyroid hormones on the calorigenic effect of CA is discussed. The question if the calorigenic effect of thyroid hormones is in some degree, mediated by the CA as proposed by Brewster and Isaac 1956, (reviews Hoch 1962, Harrison 1964), is discussed in another connection (Svedmyr, to be published.)

In experiments on both animal and man it has repeatedly been demonstrated that thyroid hormones potentiate the calorigenic effect of injected adrenaline (A) while thyroidectomy diminished it (reviews Ellis 1956, Hoch 1962, Harrison 1964 Rosenberg and Bastomsky 1965)

How this potentiation of the calorigenic effect arises is not known. Two principally different possibilities exist for the way in which thyroid hormones can in general influence the effects of the CA. The first of these is that thyroid hormones inhibit the inactivation of circulating CA so that there is a higher concentration of amine to influence the receptors, the second possibility is that thyroid hormones influence the adrenergic receptors or the reaction provoked by stimulation of these receptors. A combination of these actions is, of course, conceivable. The question of whether thyroid hormones inhibit the inactivation of circulating CA is discussed in two further studies one in experiments on man (Häggendal and Svedmyr 1966) and the other on the rat (Svedmyr and Waldeck 1965). No conclusive evidence in favour of such an effect of thyroid hormones on the CA metabolism was found in these investigations.

The calorigenic effect of CA has been assumed to be closely related to the glycogenolytic and lipolytic effects of these hormones. This subject is recently reviewed by Lundholm, Mohme Lundholm and Svedmyr (1966). In experiments on the rabbit, Lundholm (1949) found that the calorigenic effect of A could be largely ascribed to an increased production of lactic acid. The calorigenic effect appeared when the lactic acid was metabolized. The intimate relationship in the rabbit between increased lactic metabolism and the calorigenic effect of A has since been elucidated in further studies (Drury and Wick 1958, Lundholm and Mohme Lundholm 1960, Lundholm and Svedmyr 1963, 1964, 1966, Svedmyr 1966 a, Lundholm *et al* 1966). It appears that in man the calorigenic effect of A can be ascribed at least in part, to an increased lactic acid metabolism (Bearn, Billing and Sherlock 1951, Svedmyr 1966 b, c, e). In the dog A stimulated primarily the carbohydrate metabolism, while the effect on the lipid metabolism was less significant (Havel 1964). The calorigenic effect of NA seems, however, both in the dog (Havel 1964) and in man (Steinberg *et al* 1964) to be associated with an increased turnover rate and oxidation of FFA. NA stimulates the lactic acid production only to a very small extent (Lundholm 1950, Cobbold *et al* 1960, Lundholm and Svedmyr 1965). It has therefore been assumed that the calorigenic effect of NA is due partly to increased fatty acid oxidation resulting from an increase in plasma FFA and partly re-esterification of the FFA into triglycerides, which is an energy-consuming reaction (Havel 1964, Steinberg *et al* 1964). Blockade of the lipolytic effect of NA by nicotinic acid reduced its calorigenic effect in man with about 50 per cent (Havel *et al* 1964).

Thyroid hormones have been claimed to potentiate both the hyperlactacidemic effect of A (Brewster *et al* 1956) and the lipolytic effect of A and NA (Debons and Schwartz 1961, Deykin and Vaughan 1963), but Hamburger *et al* 1963 found no potentiated effect in hyperthyrotoxic patients.

It is therefore conceivable *a priori* that an increase in the calorigenic effect inter alia of the CA may be provoked by a potentiation of its effect both on the carbohydrate and on the lipid metabolism. In the rabbit the lipolytic effect of A, as reflected in the

raise of plasma FFA, was transient and not temporally correlated to the increase in oxygen consumption The effect of A on the lactic acid concentration in the blood was, however, pronounced (Svedmyr 1966 d) NA, on the other hand, had a more protracted lipolytic effect, while its effect on the lactic acid concentration in the blood was weak.

By studying the influence of thyroid hormone administration on the calorigenic effect of both A and NA in the rabbit some concept of the importance of the glycogenolytic and lipolytic effects for the potentiating influence of thyroid hormones on the calorigenic action of the CA might be obtained

In one series of experiments, the influence of thyroxinetreatment on the calorigenic, hyperglycemic, lipolytic and hyperlactacidemic effects of A and NA was studied Since it was found in these experiments that only the calorigenic and hyperlactacidemic effects of A were potentiated by the thyroxine treatment, a more detailed study was made in another series of experiments of the way in which the A action on these functions was affected by thyroidectomy, and also of how the effect of different A doses was influenced by administration of thyroxine.

Method

TABLE I The influence of adrenaline (A) and noradrenaline infusion (NA) on the oxygen the concentration of free fatty acids in plasma (FFA) on untreated (A, NA) and

		A	ATx
Mean weight kg		2 00±0 08 (n=8)	2 34±0 08 (n=8)
O_2 basal value		9 21±0 47	14 94±0 38
Mean increase over basal value	0—120 min	1 66±0 22 P< 0 001	3 31±0 26 P< 0 001
L.A. basal value		5 7±0 69	7 1±0 85
Mean increase over basal value	15 min	19 0±3 80	27 7±2 90
	60 min	43 2±3 23	69 6±6 05
	150 min	6 0±1 31	9 2±3 09
G basal value		84±3	91±5
Mean increase over basal value	15 min	117±11	61±16
	60 min	261±33	137±24
	150 min	55±13	31±9
FFA basal value		1 166±90	1 203±33
Mean increase over basal value	15 min	520±80	499±84
	60 min	−14±73	18±121
	150 min	−231±87	262±67

Time after start of the catecholamine infusion n = number of tests

P probability that the effect was due to chance

A and NA infusion during 0—60 min (0 5 μg/kg/min)

Results

1 *The effect of thyroxine treatment on the calorigenic, hyperglycemic hyperlactacidemic and lipolytic effects of adrenaline and noradrenaline*

Thyroxine treatment increased the basal oxygen consumption by an average of 60 %. The basal lactic acid concentration in the blood increased very slightly after thyroxine treatment, while the glucose content of the blood and the concentration of FFA in plasma were unchanged Table I Fig 1

The calorigenic effect of 0 5 μg A per kg per min given for 60 min was increased twofold and with statistical significance by treatment with thyroxine in comparison with the effect in untreated animals Table I) The A effect on the oxygen consumption was calculated in ml kg min increase over the basal value This method of calculating was used because the basal oxygen consumption was different in untreated and

consumption (O_2), on the blood concentration of lactic acid (L.A.) and glucose (G) and on thyroxinetreated rabbits (ATx, NATx)

Change ATx A	NA	NATx	Change NATxNA
0 34±0 11	2 60±0 26 (n 7)	2 48±0 11 (n 6)	0 12±0 28
5 73±0 60 P< 0 001	8 33±0 28	13 83±0 53	5 50±0 57 P< 0 001
1 65±0 37 P< 0 001	0 61±0 10 P< 0 001	0 65±0 19 P< 0 02	0 04±0 22
1 4±1 10	6 2±0 65	6 9±1 14	0 7±1 33
8 7±4 79	3 6±1 49	0 5±0 20	−3 1±1 51
26 4±6 86 P< 0 005	1 5±0 85	2 4±1 08	0 9±1 38
3 2±3 4	1 0±0 92	1 6±0 73	0 6±1 18
+8±6	90±3	89±6	1 0±7
−56±19 P< 0 02	34±6	25±8	−9±10
−124±37 P< 0 005	48±4	41±11	−7±12
24±16	13±4	11±9	−2±11
43±97	1 041±96	1 073±171	32±197
−21±116	569±89	385±162	184±185
+31±141	506±132	369±99	137±164
−31±110	125±74	25±80	+150±109

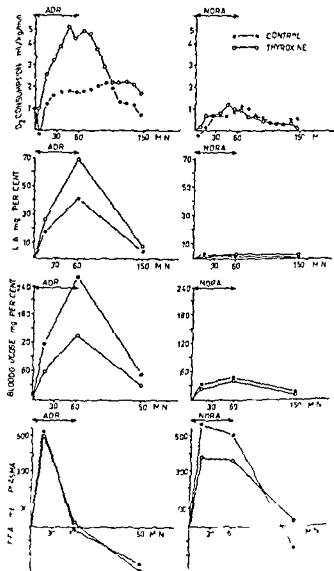
Tx treatment 1 mg/day during 7 days

O_2 ml/kg/min, L.A. mg per 100 ml glucose mg per 100 ml, FFA μE/l

thyroxine-treated animals A percental calculation of the oxygen consumption would underestimate the A effect in thyroxine treated animals in comparison with the control animals

The effect of A on the lactic acid concentration in the blood was also increased after thyroxine treatment (Table I Fig 1) On the other hand the increase in glucose concentration in the blood was significantly smaller in the thyroxine-treated animals No definite change of the A effect on the plasma FFA was noted

NA given in a dose of 0 5 μg/kg/min for 60 min had a markedly weaker effect on the oxygen consumption lactic acid and glucose concentrations in the blood than A The effect on the plasma FFA on the other hand was considerably more prolonged after NA infusion Thyroxine treatment did not influence with statistical certainty any of the metabolic effects of NA



[illegible] ...genic [illegible] hyperlactic[illegible] [illegible]eriments on [illegible] Each point [illegible]

These experiments indicate that thyroxine treatment potentiated the calorigenic effects of A but not NA in the rabbit. The elevating effect of A on the lactic acid concentration in the blood was also augmented by thyroxine administration. The parallelism between the effects of thyroxine on the calorigenic and hyperlactacidemic action of A has been studied more closely in subsequent experiments.

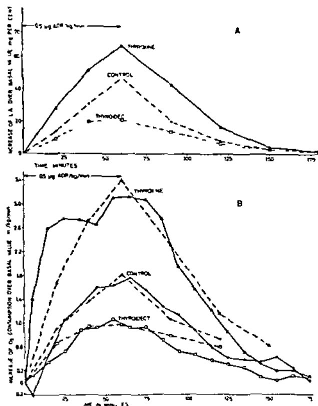


Fig 2 A The effect of adrenaline infusion (0 5 µg/kg/min for 60 min) on the lactic acid (L.A.) content of the blood in untreated (control, n = 8), thyroidectomized (n = 10) and thyroxine treated (n = 8) rabbits Mean increase over basal value

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0 43 Y = increase of oxygen consumption over basal value in ml kg/min. X = increase of lactic acid content in the blood over basal value in mg %

2 The effect of 0 5 µg adrenaline kg min on oxygen consumption and lactic acid concentration in the blood in thyroxine-treated thyroidectomized and untreated rabbits

As may be seen in Fig 2B and Table II the calorigenic effect of A was reduced after thyroidectomy and increased after thyroxine administration compared with the effect in the untreated animals The mean effect during the period 0—120 min after the start of the infusion was reduced in the thyroidectomized animals to about 60 % of the value in the untreated animals and increased to about 220 % of this value in the thyroxine-treated animals These change were statistically significant

TABLE II The effect of 0.5 µg adrenaline/kg/min (A) on oxygen consumption and lactic acid concentration in the blood Tests in untreated (A), thyroxine treated (ATx) and thyroidectomized (AThe) rabbits (Symbols as in Table I)

	A (n = 7)	ATx (n = 8)	AThe (n = 10)	Change ATx A	Change A AThe
Mean weight kg	3.0 ± 0.17	3.0 ± 0.13	3.3 ± 0.3	0.0 ± 0.25	0.3 ± 0.3
Oxygen consumption					
Basal value	8.84 ± 0.26	12.65 ± 0.42	6.90 ± 0.15	3.81 ± 0.49 P < 0.001	1.96 ± 0.3[illegible] P < 0.001
Mean increase over Basal value 0 120 min	1.09 ± 0.10 P < 0.001	2.40 ± 0.16 P < 0.001	0.64 ± 0.14 P < 0.005	1.51 ± 0.18 P < 0.001	0.45 ± 0.17 P < 0.02
Lactic acid					
Basal value	6.7 ± 0.5	9.7 ± 0.5	5.8 ± 0.8	3.0 ± 0.7 P < 0.001	0.9 ± 1.0
Mean increase over Basal value					
after 20 min	14.2 ± 3.3	28.2 ± 2.9	9.3 ± 2.3	14.0 ± 4.6 P < 0.01	4.9 ± 4.2
40 min	31.3 ± 5.3	51.6 ± 2.8	19.9 ± 5.0	20.3 ± 6.0 P < 0.005	11.4 ± [illegible]3
60 min	47.0 ± 5.2	66.8 ± 3.2	20.9 ± 4.6	19.7 ± 6.2 P < 0.01	26.0 ± [illegible]1 P ~ 0.00[illegible]
90 min	20.4 ± 2.5	42.6 ± 3.7	13.9 ± 3.0	22.2 ± 4.4 P < 0.001	6.5 ± 3.8
120 min	8.2 ± 1.7	16.6 ± 2.6	6.2 ± 1.7	8.5 ± 3.0 P < 0.02	2.0 ± 2.3

The influence of thyroidectomy and thyroxine administration on the effect of A on the lactic acid concentration in the blood is shown in Fig 2 A and Table II In the thyroidectomized animals the increase of this concentration was smaller throughout the infusion than in the untreated animals, and after 60 min infusion this difference was significant After thyroxine treatment the increase of the lactic acid concentration in the blood was higher than in the normal animals, this increase being statistically significant

3 The effect of 0.05 µg adrenaline kg/min on oxygen consumption and lactic acid concentration in the blood in thyroxine treated and untreated rabbits

In previous experiments Lundholm and Svedmyr 1964) an increasing calorigenic effect of A was observed in the dose region of 0.05—0.5 µg kg/min given for 60 min. On the other hand 2 µg of A kg min did not have a markedly stronger effect than 0.5 µg (Lundholm 1949 I therefore investigated the influence of thyroxine treatment on the effect of a small A dose 0.05 µg kg/min) with the expectation that a potentiation of the A effect might be most marked in this dose region After thyroxine treatment a possible tendency to potentiation of the calorigenic effect of this small A dose was

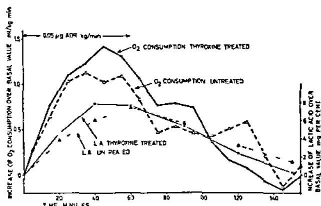


Fig 3 The effect of adrenaline infusion (0 05 μg/kg/min for 60 min) on the lactic acid (L.A) content of the blood in untreated and thyroxine-treated rabbits Mean increase over basal value of 8 animals

TABLE III The effect of 0 05 μg adrenaline/kg/min on the oxygen consumption and the lactic acid concentration in the blood in untreated (A) and thyroxine treated (ATx) rabbits (Symbols as in Table I)

		A (n=8)	ATx (n=8)	Change ATx A
Mean weight kg		3 08±0 29	3 36±0 17	0 28±0 34
Oxygen consumption				
Basal value		9 62±0 21	13 83±0 39	4 21±0 44 P< 0 001
Mean increase over basal value	0—120 min	0 71±0 06 P< 0 001	0 85±0 18 P< 0 005	0 14±0 19
Lactic acid				
Basal value		7 0±0 47	8 9±0 40	1 9±0 62 P< 0 01
Mean increase over basal value after	20 min	3 8±8 0	4 6+1 0	0 8±1 3
	40 min	6 1±1 0	7 9±1 5	1 8±1 6
	60 min	7 5±1 2	7 7±1 7	0 2±1 8
	90 min	5 6±1 5	5 4±0 5	0 2±1 6
	120 min	3 3±0 7	2 4±0 9	0 9±1 2

noted (Fig 3, Table III) but this increase was not statistically significant The increase in the lactic acid concentration in the blood after A infusion was not influenced with certainty by thyroxine after 60 min A infusion the increase was identical in the thyroxine treated and untreated animals (Fig 3 Table III

Discussion

The administration of thyroxine in the rabbit potentiated the calorigenic effect of A but not that of NA However, only the effect of a large A dose was potentiated with certainty, but not that of a smaller dose Parallel with the calorigenic effect, thyroxine increased the effect of A on the lactic acid concentration in the blood, while the hyperglycemic effect was decreased and the effect on the plasm FFA remained unchanged

The potentiation, after thyroxine administration, to the increase in the lactic acid concentration in the blood caused by A may be due either to an increase in the rate of lactic acid production in the tissues or a decrease in lactic acid elimination In another investigation (Svedmyr 1966 a) the effect of lactate infusion on the lactate concentration in the blood and on the oxygen consumption was studied in thyroidectomized, normal and thyroxine treated rabbits It was found that the rate of elimination of lactate was increased after thyroxine administration but unchanged after thyroidectomy It may be assumed therefore that the potentiation of the lactic acid increasing effect of A which was induced by thyroxine was due to increased lactic acid production in the tissues. For the same reason it may be assumed that the decreased effect after thyroidectomy was due to reduction of the lactic acid producing effect of A

The equations used in the above-mentioned study for correlating the increase in oxygen consumption with the increase in lactic acid concentration in the blood may be seen in the text of Fig 2B The increase in oxygen consumption which might be expected to take place as a result of lactic acid metabolism in the A tests was calculated by means of these equations from the actual increase in the lactic acid concentration in the blood observed in the different experimental groups in the present investigation. The time course of this expected oxygen consumption in the different groups is shown by the dotted lines in Fig 2B As may seen in the figure, the "expected" and experimentally obtained curves for the oxygen consumption corresponded closely in the different groups By planimetry of the areas under the experimentally obtained and the expected curves for the period 0—120 min after the start of the A infusion, that proportion of the total calorigenic effect of the A which might be due to an increase in lactic acid metabolism was estimated In the untreated animals the area under the experimentally obtained curve was 100 per cent of that under the expected curve In the thyroidectomized animals the corresponding percentage was 102 and in the thyroxine treated animals 96

The importance of the CA effect on lipid metabolism for the potentiation of the calorigenic effect after thyroxine treatment was probably of minor importance in these experiments Thyroxine treatment had no influence on the FFA increasing effects in plasma of either A or NA Neither was the calorigenic effect of NA potentiated by thyroxine treatment despite the fact that its influence on the plasma FFA was more marked than that of A These results suggest indirectly that an increase lipid metabolism was of little importance for the potentiation of the calorigenic effect of A after thyroxine treatment

The hyperglycemic effect of A was diminished by thyroxine treatment in these experiments This is in direct contrast to most findings of other workers (Hoch, 1962, Harrison 1964 Danowski *et al* 1964 who have shown that thyroxine increases the effect of A on the blood sugar It is probable that this difference is due to the fact that my experiments were performed on fasted rabbits whose glycogen store in the liver was probably low and decreased further as a result of the thyroxine treatment The

magnitude of the glycogen stores in the liver has been suggested to be of importance for the magnitude of the hyperglycemic response of A (Burn and Marks 1925) In experiments on fed animals the hyperlactacidemic effect, for example, of A was considerably stronger than in fasted animals (Lundholm and Svedmyr 1966) It is also possible however, that the rate of glucose oxidation was increased in the thyroxine-treated animals, which could have contributed towards reducing the hyperglycemic effect of A

It appears that in rabbits a large part of the calorigenic effect of A under different degrees of thyroid hormone influence may be explained as being due to variations in lactic acid production and metabolism

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References

Bearn A G, B Billing and S Sherlock The effect of adrenaline and noradrenaline on hepatic blood flow and splanchic carbohydrate metabolism in man. *J Physiol* (Lond) 1951 *115* 430—441

Bergmeyer H and E Bernt, D Glucose Bestimmung mit Glucose-Oxydase und Peroxydase In *Methoden der Enzymatischen Analyse* (H U Bergmeyer ed) Verlag Chemie Weinheim 1962 123—130

Brewster W R Jr J P Isaacs P F Osgood and T L. King The hemodynamic and metabolic interrelationships in the activity of epinephrine norephinephrine and the thyroid hormones [illegible]

B[illegible]

C[illegible]

D[illegible]

D[illegible]

Deykin D and M Vaughan, Release of free fatty acids by adipose tissue from rats treated with [illegible]

D[illegible] *J Physiol*

E[illegible] 1956, *8* 463—[illegible]2

Friedmann T E and J B Graeser The determination of lactic acid *J biol Chem* 1933 *100* 291—308

Harrison T S Adrenal medullary and thyroid relationships. *Physiol Rev* 1964 *44* 161—185

Hamburger J R W Smith Jr and J M Miller Effects of epinephrine on free fatty acid mobilization in hyperthyroid and hypothyroid subjects. *Metabolism* 1963 *12* 821—878

Havel R J Catecholamines In *Lipid Pharmacology* (R Paoletti ed Acad Press New York 1964 pp 357—380

Havel, R. J L. A Carlson L G Ekelund and A Holmgren Studies on the relation between mobilization of free fatty acids and energy metabolism in man Effects of norepinephrine [illegible]

[illegible] ntent 05 [illegible]

LUNDHOLM L., The effect of l noradrenaline on the oxygen consumption of resting animals. *Acta physiol scand* 1950 *21* 195—204

LUNDHOLM L. and E. MOHME LUNDHOLM The action of adrenaline on carbohydrate metabolism in relation to some of its pharmacodynamic effects In *Adrenergic mechanisms* Ciba Foundation Symposium ed by J R Vane G E V Wostenholme and C. M O Connor pp 305—321 Churchill London, 1960

[illegible]

LUNDHOLM L. and N SVEDMYR Blockade of the lactic acid-stimulating and calorigenic effects of adrenaline or isoprenaline by chloroisoprenaline *Acta pharm* (*Kbh*) 1963 *20* 303—3[illegible]

LUNDHOLM L. and N SVEDMYR Respiratory response to adrenaline and noradrenaline in man. *Acta physiol scand* (in press)

LUNDHOLM L. and N SVEDMYR, The calorigenic effect of adrenaline in fasted and fed rabbits. (Unp obsers)

MOHME E. Effect of d hydroergotamine on the oxygen consumption. Experiments on cats. *Acta physiol scand* 1949 *18* 63—67

ROSENBERG I N and C H BASTOMSKY The Thyroid. Annu Rev Physiol 1965 *27* 7[illegible]—10[illegible]

STEINBERG D. P J NESTEL, R ELSEWORTH R BONIRK and R A THOMPSON Calorigenic effect of norepinephrine correlated with plasma free fatty acid turnover and oxidation. *J clin Invest* 1964 *43* 167—176

SVEDMYR, N Lactate elimination and oxidation in thyroidectomized untreated and thyroxine-treated rabbits *Acta physiol scand* 1966 a. *66* 67—71

SVEDMYR N The influence of triiodothyronine treatment on some of the metabolic effects of adrenaline and noradrenaline in experiments on man. *Acta physiol scand* 1966 b (In press)

SVEDMYR N The influence of infusion of l lactate on the oxygen consumption in man. Acta physiol scand 1966 c In press)

SVEDMYR N Blockade of the calorigenic and some other metabolic effects of adrenaline by pronethalol Acta pharm tox. 1966 d. (In press)

SVEDMYR N Studies on the mechanism of the calorigenic effect of adrenaline on man. Acta *physiol scand* 1966 *e* In press)

SVEDMYR N and B WALDECK The effect of l thyroxine treatment on the metabolism of tritium-labelled catecholamines in the rat *Acta pharm. tox* 1965 *23* 225—230

TRENDELENBURG L Thyroid and hyperglycemia produced by adrenaline and noradrenaline *Brit J Pharmacol* 1953 *8* 454—460

TROUT D L. E H ESTES and S J FRIEDBERG Titration of free fatty acids of plasma A study of current methods and a new modification. *J Lipid Res* 1960 *1* 199—201

WILSON W R F O THEILEN AND F W FLETCHER Pharmacodynamic effects of beta adrenergic receptor blockade in patients with hyperthyroidism *J clin Invest* 1964 *43* 1697—1703

Acta physiol. scand. 1966. 66. 269—277

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The Effect of Bradykinin, Kallidin and Eledoisin upon the Pulmonary Vascular Bed of an Isolated Blood-Perfused Rabbit Lung Preparation

By

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Received 28 June 1965

Abstract

as e.g. 5 hydroxytryptamine responses were reduced to the same extent

Bradykinin, kallidin and eledoisin cause a marked general vasodilatation in the systemic circulation in all species examined (Rocha e Silva, Beraldo and Rosenfeld 1949, Lecomte and Troquet 1960, Kontos *et al.* 1964 a, b). A local vasodilator effect of the kinins, and mainly of bradykinin, has been reported also for many areas or organs of the systemic circulation, such as the submandibular salivary gland (Hilton and Lewis 1955 a, b, 1956), the skin (Fox and Hilton 1958, Elliot, Horton and Lewis 1960), striated muscle (Kjellmer and Odelram 1965), the myocardium (Bergamaschi and Glasser 1963), the kidney (Barer 1963), and the central nervous system (Carpi and Corrado 1961). Vasoconstrictor responses to bradykinin have been reported, however, for certain parts of the systemic circulation, such as the rabbit ear (Guth, Cano and Jamarillo 1963) and the A-V anastomoses of the finger pulp (Burch and Pasquale 1962).

The effect of the plasma kinins in the pulmonary vascular bed is not quite clear. The kinins seem to cause pulmonary vasoconstriction in most of the species examined

Lecomte and Troquet (1960) thus found a vasoconstrictor effect of bradykinin in the perfused rabbit lung preparation, using Locke's solution as a perfusate. Gerzmeyer and Spitzbarth (1961) found a rise in the pulmonary arterial pressure upon i.v. injection of bradykinin in man, and Klupp and Konzett (1963) found the same in cat and guinea pig. The last authors also found the pulmonary arterial pressure to increase without a concomitant rise in the minute volume, upon injection of bradykinin in a guinea pig heart lung preparation.

Some reports seem, however, to indicate a vasodilator effect of the kinins in the pulmonary vascular bed under certain conditions. Freitas, Faraco and Azevedo (1964) thus found a decreased pulmonary vascular resistance upon i.v. infusions of bradykinin in man, and injections of bradykinin caused a small pulmonary vasodilatation in an isolated blood-perfused lung preparation of the dog (Waaler 1961).

In the present investigation we have attempted to analyse the vascular effects of bradykinin, kallidin and eledoisin upon pulmonary vessels under stable conditions with satisfactory control of perfusion and ventilation, and with whole blood as a perfusate. Isolated rabbit lungs were used as test organ.

The action of plasma kinins on a vascular bed might be an indirect one. Lecomte, Troquet and Dresse (1961) suggested that i.v. injected bradykinin is releasing catecholamines from the adrenal medulla in the rabbit. Feldberg and Lewis (1964) have later demonstrated that adrenaline is released from the suprarenals of the cat upon i.a. injection of bradykinin. A main problem in the present investigation has therefore been to see if the kinins were acting upon the pulmonary vascular bed through a liberation of other known vasoactive agents, such as noradrenaline, 5-hydroxytryptamine or histamine. These substances might conceivably be liberated either from the lung or from the cellular elements of the perfusate.

A preliminary report of the results obtained has been given elsewhere (Hauge, Lunde and Waaler 1964).

Materials and methods

Preparation. Isolated rabbit lungs were perfused with heparinized homologous blood under conditions of constant volume inflow. Positive pressure ventilation with 4 % CO_2 in air was used. The pulmonary arterial pressure (PAp) and the ventilation overflow (VOF) were recorded continuously. The left atrial pressure (LAp) was kept constant in each experiment, at a level varying from 3 to 6 cm of water. The temperature of the perfusate was kept constant at about 37–38° C and pH in the perfusate was controlled throughout the experiments. A more detailed description of the preparation with its perfusion and ventilation arrangements is given in a previous paper (Hauge, Lunde and Waaler 1965 a).

Drug addition. The various drugs were tested at intervals during the most stable periods of the perfusion, sometimes after the addition to the perfusate of tri-cresol (Hauge et al. 1965 a). Fresh dilutions of the drugs in a sodium chloride solution (0.9 g/100 ml) were made up before each experiment. Drug injections were carried out into the pulmonary arterial tubing near the pulmonary artery in volumes of 0.5 ml, using a syringe with a very fine needle. Infusions [illegible]

[illegible]

perfusate.

List of drugs used

Bradykinin (Synthetic bradykinin, BRS 640, Sandoz A. G.), Kallidin (Synthetic Kallidin, KL 698, Sandoz A. G.), Eledoisin (Synthetic Eledoisin, ELD 950, Sandoz A. G.), Acetylcholine (Acetylcholine chloride cryst., F. Hoffmann-La Roche & Co. A. G.), 5-hydroxytryptamine [illegible]

Vent overflow (ml) 15 10 5 0

PAp (cm saline) 25 24 23 22

30 sec

530 pm 538 546 554
B K K B
μg 2 1 1 2

Fig 1 *Effect of injections into the pulmonary artery of equipotent doses of bradykinin (B) and kallidin (K) at regular intervals of 8 min*

Isol[illegible]
at 1
4 cr
PAp

amine (Serotonin creatinine H_2SO_4 H_2O complex, California Foundation of Biochemical

Inhibitors

[illegible] 'Deseril®, Sandoz A G)
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[illegible] (Regitin®, Ciba Société

Results

Doses of bradykinin, kallidin or eledoisin which were sufficiently large to give any response at all, invariably caused vasoconstriction when injected or infused into the pulmonary arterial tubing The minimal effective doses of the three polypeptides varied from one preparation to another, and were also apparently somewhat dependent on the flow and pressure situation A smaller dose could often suffice in a situation with a reduced blood flow Changes in pH from 7 2 to 7 9 induced by alterations in the CO_2 content of the ventilation gas affected the responses to kinin injections to a surprisingly small degree

In a series of experiments the minimal effective doses on pulmonary arterial injections were found to be as follows for eledoisin 0 01—1 10 $^{-8}$g, for kallidin 0 25—2 5 10 $^{-8}$g and for bradykinin 0 5—5 10 $^{-8}$g

Bronchoconstriction was not seen following pulmonary arterial injections of the kinins The bronchial vessels however, were not perfused in these preparations

Among the other substances which were tested by arterial injections in this preparation, noradrenaline and 5 hydroxytryptamine in doses of from 5 to 50 10 $^{-8}$g also caused



Fig 2 *Effect of repeated injections into the pulmonary artery of a constant dose of kallidin (K) at decreasing intervals*

IPL rabbit ♂ 2 5 kg First three intervals between injections were 6 min the next two intervals were 4 min and the following two intervals 3 min The last two injections were carried out after pauses of 15 and 26 min, respectively Perfusion (constant volume inflow) started at 12 13 p m BF 200 ml min LAp 4.5 cm H_2O TV decreased from 24 to 21 ml during the period illustrated Abbreviations as in Fig 1

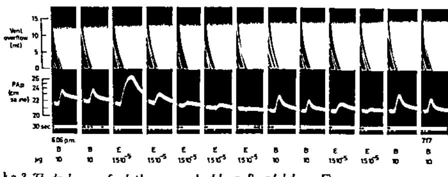
vasoconstrictor responses Histamine, in doses of from 5 to 20 10 $^{-6}$g gave marked vasoconstriction in most experiments, but minute responses or even vasodilatation in some other experiments Adrenaline had a marked vasodilator effect in most preparations Dopamine in doses which were 10 times larger than effective doses of noradrenaline, had practically no effect upon the pulmonary vascular bed Acetylcholine in doses down to 0 5 10 $^{-6}$g caused marked vasoconstrictor responses, as has been shown by Euler (1932) With the exception of acetylcholine the three polypeptides were therefore the most potent vasoconstrictor agents tested when evaluated on a weight basis as well as on a molar basis

Responses to injected doses of the various substances tested can be seen in Figs 1 to 7 The relative potency of the various substances differed somewhat, however, from one preparation to another

Tachyphylaxis Fig 1 shows the responses to equipotent doses of bradykinin and kallidin injected at regular intervals On a weight basis kallidin was some 40 to 80 % more potent than bradykinin The moderately diminishing responses seen on the figure indicate some tachyphylaxis and apparently a cross tachyphylaxis between bradykinin and kallidin

The degree of tachyphylaxis which developed on repeated injections of kallidin is illustrated in Fig 2 A standard dose of kallidin was here injected at intervals which were reduced from 6 to 4 and further to 3 min This was the shortest interval which would allow the PAp-level to return to its pre injection value On such a series of injections the kallidin responses were reduced by some 70 % The final two injections were carried out after pauses of 15 and 26 min respectively, and the responses then recovered to the initial value The size of the responses is obviously closely related to the injections intervals

In Fig 3 the responses to standard doses of bradykinin and eledoisin can be compared Injections were carried out at regular intervals Eledoisin was several thousand times more potent than bradykinin in this particular preparation A very marked tachyphylaxis for eledoisin developed and there was practically no response to a fifth injection The response to bradykinin was by then not reduced nor was it reduced after two more



in Fig 1

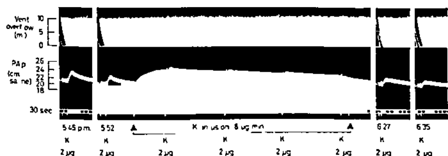


Fig 4 *Effect of kallidin (K) injections into the pulmonary artery before during and after a kallidin infusion*

IPL, rabbit, ♀ 4.8 kg Perfusion (constant volume inflow) started at 11.32 a.m. BF 220 ml/min. LAp 3.5 cm H_2O TV decreased from 15 to 14 ml during the period illustrated Autologous blood was used as a perfusate in this perfusion Abbreviations as in Fig 1

eledoisin injections This indicates that there is no cross-tachyphylaxis between eledoisin and bradykinin If an interval of time was allowed to elapse after the responses to eledoisin injections had disappeared the responses to this substance also returned (Fig 3), but notably slower than did responses to bradykinin or kallidin (Fig 2) This may be related to the fact that the inactivation of eledoisin is much slower in rabbit blood than is the inactivation of bradykinin or kallidin When 0.5 10 $^{-6}$g bradykinin was added to 1 ml of heparinized rabbit whole blood at 37° C, no bradykinin activity could be detected on the isolated rat uterus preparation after 2 min of incubation. When 0.4 10 $^{-10}$g of eledoisin was incubated with 1 ml of blood at 37° C however some 30 % of the initial activity was still present after 12 min as tested in our preparation The whole activity was lost after about 20 min of incubation

In Fig 4 the development of tachyphylaxis to kallidin is demonstrated in another way Here kallidin was infused near the pulmonary artery over a period of 26 min Injections of a standard dose of kallidin were carried out before during and after this infusion The pulmonary vascular bed shows decreasing responses both to the infusion itself,

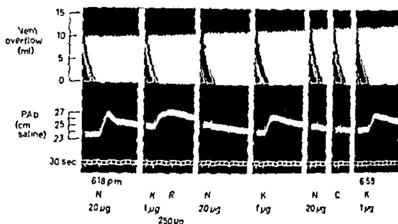
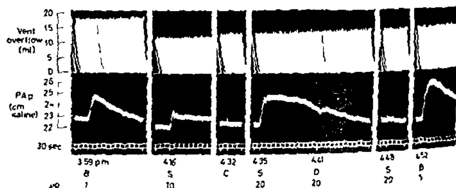


Fig 5 *Effect o injections into the pulmonary artery of noradrenaline (N) and kallidin (K) at intervals*

ustant volume inflow) m 17 to 14 ml during



S) *before and after the*

ant volume inflow 19 to 15 ml during the period illustrated At 4 05 p m the stroke volume of the ventilation pump was reduced. Abbreviations as in Fig 1

and still more to the superimposed injections of kallidin After cessation of the infusion, the responses to injections increased nearly to their pre-infusion level

Infusion of the same dose of kallidin into the venous reservoir over a further period of 25 min gave no response from the pulmonary vascular bed. This indicates that the peptide, when added in this way was already inactivated when reaching the preparation some 30—40 sec after being added to the blood The responses to pulmonary arterial injections of kallidin during this period with infusion into the reservoir were not reduced in size Thus the fresh products from kallidin inactivation did not seem to affect the kinin receptors in the pulmonary vascular bed

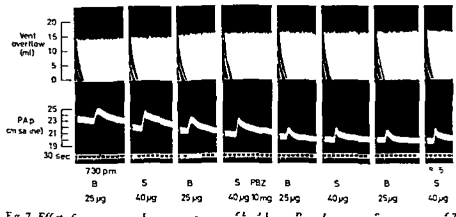


Fig 7 Eff[illegible]

The mechanism of the vascular effect of bradykinin and kallidin The possibility that the plasma kinins might act indirectly and through a release of other vasoconstrictor agents was considered Lung tissue is known to contain catecholamines, especially dopamine (Euler and Lishajko 1957, Schumann 1958) and rabbit blood platelets are very rich in 5-hydroxytryptamine and histamine (Humphrey and Jaques 1954)

Fig 5 shows the responses to injections of standard doses of noradrenaline and kallidin before and after the addition to the perfusate of the inhibitor Regitin® The effect of noradrenaline was completely inhibited after the addition while that of kallidin was unaffected Fig 6 shows that when the response to 5-hydroxytryptamine injections was inhibited after the addition of Deseril® bradykinin was still as active as before Similarly the effect of injected histamine could be completely inhibited by addition of Anthisan® without the vasoconstrictor responses to bradykinin or kallidin being affected at all Finally the addition to the perfusate of 1mg of atropine could be shown to have no effect on responses to intra arterially injected plasma kinins

Addition to the perfusate of phenylbutazone or sodium salicylate Phenylbutazone has been reported to cause marked inhibition of the responses to bradykinin in the isolated rabbit lung (Lecomte and Troquet 1960 In Fig 7 the inhibitory effect of a large dose of this substance is demonstrated Sodium salicylate had a similar effect. The responses to injected bradykinin and kallidin were reduced by some 30—60 % only and apparently nonspecifically as for example the response to 5-hydroxytryptamine was reduced to the same extent Addition of more phenylbutazone had no further effect on the responses to injected kinins

Discussion

On a weight basis and still more on a molar basis, bradykinin kallidin and eledoisin were more potent vasoconstrictors in this lung preparation than were 5-hydroxytrypt amine noradrenaline and histamine The difference that can be demonstrated between the vasoconstrictor effect of these polypeptides in the pulmonary circulation and their

vasodilator effect in the systemic circulation is thus striking, and of considerable principal interest At present we can offer no explanation for this difference

The vasoconstrictor effect of the three polypeptides is apparently not mediated via liberation into the blood of the other known vasoconstrictor agents investigated The constrictor effects of 5 hydroxytryptamine, noradrenaline and histamine injections could thus be efficiently inhibited without the polypeptide induced vasoconstriction being affected

In isolated lung vessels of the guinea pig bradykinin has been shown to cause more marked constriction of post-capillary vessels than of precapillary vessels (Moog and Fischer 1964) Using the isolated, perfused rabbit lung preparation described in this publication, Hauge, Lunde and Waaler (1965 b) have shown that infusion into the pulmonary artery of bradykinin causes no detectable increase in capillary pressure as judged from the weight changes in the preparation This indicates that the vasoconstrictor action of bradykinin is occurring at precapillary sites in the pulmonary vascular bed of the rabbit Postcapillary vessels are also reported to have a very poorly developed media in the rabbit lung, whereas the same vessels have great medial thickness in the guinea pig (Takino 1932)

Eledoisin was several hundred times more potent than the two other peptides in our preparation This is in agreement with the findings of Kovalcik (1963) who found a very great sensitivity to eledoisin in isolated strips from rabbit pulmonary artery

Bradykinin and kallidin showed cross tachyphylaxis, indicating a common site o [illegible] ucture [illegible] wever [illegible] hylaxis indicates that eledoisin has a different site of action at the receptor level This is perhaps not surprising in view of the marked structural difference between eledoisin on the one hand and bradykinin and kallidin on the other

Phenylbutazone and sodium salicylate cannot be used as plasma kinin inhibitors in our preparation The inhibition caused by these substances was moderate and apparently nonspecific as the effects of different other vasoconstrictors were equally affected

We want to [illegible] generous supplies [illegible] earch Fellow [illegible] pport from th [illegible] vascular [illegible]

References

BAKER G R The action of vasopressin a vasopressin analogue PLV, oxytocin angiotensin, bradykinin and theophylline ethylene diamine on renal blood flow in the anaesthetized rat. J Physiol Lond 1963 16? 62 72

BERGAMASCHI M and A H GLASSER Effect of the endecapeptide eledoisin on the coronary blood flow Comparison with nitroglycerin bradykinin and epinephrine in the dog Circulat Res 1963 13 329 335

BURCH G E and N P DE PASQUALE Bradykinin digital blood flow and the arteriovenous anastomoses. Circulat Res 1962 10 105 115

CARPI, A and A P CORRADO Cerebral vascular action of bradykinin in the dog Experientia (Basel) 1961 17 326 327

ELLIOT D F, E W HORTON and G P LEWIS Actions of pure bradykinin *J Physiol* (Lond) 1960 *153* 473—480

EULER, U S. von A vaso-constrictor action of acetylcholine on the rabbits pulmonary circulation. *J Physiol* (Lond) 1932 *72* 271—278

EULER U S. von and F LISHAJKO, Dopamine in mammalian lung and spleen. *Acta physiol pharmacol. neerl* 1957 *6* 295—303

FELDBERG W and G P LEWIS, The action of peptides on the adrenal medulla Release of adrenaline by bradykinin and angiotensin *J Physiol* (Lond) 1964 *171* 98—108

FOX R H and S M HILTON, Bradykinin formation in human skin as a factor in heat vasodilatation *J Physiol* (Lond) 1958 *142* 219—232

FREITAS F M DE E Z FARACO and D F DE AZEVEDO, General circulatory alterations induced by intravenous infusion of synthetic bradykinin in man *Circulation* 1964 *29* 66—70

GERZMEYER, E F und H SPITZBARTH Über Kreislaufswirkungen von synthetischem Bradykinin beim Menschen und beim wachen Hund *Klin W schr* 1961 *39* 1227—1233

GUTH P S, G CANO and J JAMARILLO The effect of bradykinin on vascular smooth muscle *Ann N Y Acad Sc* 1963 *104* 69—73

HAUGE, A, P K M LUNDE and B A WAALER The effect of bradykinin kallidin and eledoisin upon the pulmonary vascular bed of isolated blood-perfused rabbit lungs *J Physiol* (Lond) 1964 *173* 33—34 P

HAUGE A., P K M LUNDE and B A WAALER Vasoconstriction in isolated blood perfused rabbit lungs and its inhibition by cresols *Acta physiol scand* 1966 *66* 226—240

HAUGE A, P K M LUNDE and B A WAALER Bradykinin and pulmonary vascular permeability in isolated blood perfused rabbit lungs *Biochem Pharmacol* 1965 b *14* 1393 (abstract no 19)

HILTON, S M and G P LEWIS The cause of the vasodilatation accompanying activity in the submandibular salivary gland *J Physiol* (Lond) 1955 a *128* 235—248

HILTON S M and G P LEWIS The mechanism of the functional hyperaemia in the submandibular salivary gland *J Physiol* (Lond) 1955 b *129* 253—271

HILTON S M and G P LEWIS The relationship between glandular activity bradykinin formation and functional vasodilatation in the submandibular salivary gland *J Physiol* (Lond) 1956 *134* 471—483

HUMPHREY, J H and R JAQUES The histamine and serotonin content of the platelets and polymorphonuclear leucocytes of various species *J Physiol* (Lond) 1954 *174* 305 310

KJELLMER I and H ODELRAM The effect of some physiological vasodilators on the vascular bed of skeletal muscle *Acta physiol scand* 1965 *63* 94—102

KLUPP H und H KONZETT Zur Wirkung von Bradykinin und Kallidin auf den Druck in der Arteria pulmonalis *Naunyn Schmiedeberg s Arch exp Path Pharmakol* 1963 *246* 19—20

KONTOS H A J H MAGEE W SHAPIRO and J L PATTERSON JR General and regional circulatory effects of synthetic bradykinin in man *Circulat Res* 1964 *14* 351 356

KONTOS H A W SHAPIRO H P MAUCK and J L PATTERSON JR General and regional circulatory effects of synthetic eledoisin in man *J appl Physiol* 1964 *19* 113—116

KOVALCIK V Effect of bradykinin and eledoisin on isolated vascular smooth muscle *Biochem Pharmacol* 1963 *12* (Suppl 18) (abstract no 63a

LECOMTE J et J TROQUET Antagonisme entre bradykinine et phenylbutazone chez le lapin *C R Soc Biol* (Paris 1960 *154* 1115 1117

LECOMTE J J TROQUET et A DRESSE Stimulation medullo-surrenalienne par la bradykinine *Arch int Physiol* 1961 *69* 89 91

MOOG E und J FISCHER Vergleich der Wirkung verschiedener biogener Amine und Polypeptide auf den arteriellen und venosen Lungenkreislauf *Naunyn-Schmiedeberg s Arch exp Path Pharmak* 1964 *247* 3[illegible]

ROCHA E SILVA M W T BERALDO and G ROSENFELD Bradykinin a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin *Amer J Physiol* 1949 *156* 261 73

SCHUMANN H J Uber der Hydr[illegible] und Noradrenalingehalt der Lunge *Naunyn Schmiedeberg s Arch exp Path Pharmak* 1958 *234* 28[illegible] 290

TARINO M Vergleichende Studien uber die histologische Struktur der Arteriae und Venae pulmonales die Blutgefassnerven der Lunge und die Nerven der Bronchien bei verschiedenen Tierarten besonders uber die Beziehung der Blutgefassnerven zu glatten Muskeln der Blutgefasse III Mitteilung [illegible] Med [illegible] 193[illegible] 1933 *15* 321 (Cit Handbook of Physiology Section [illegible] American Physiological Society Washington D C. 1963 *11* p 1674

WAALER B A The effect of bradykinin on an isolated perfused dog lung preparation *J Physiol* (Lond 1961 [illegible]

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Effects of Acute Heart Failure and Administration of Ouabain on Cardiac Catecholamine Uptake

By

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Abstract

Oliverio A and H h Wang *Effects of acute heart failure and administration of ouabain on cardiac catecholamine uptake* Acta physiol scand 1966 *66* 278—281 — The cardiac catecholamine uptake of tracer doses of tritiated noradrenaline (^{3}H-dl NA) was studied using guinea pig heart lung preparations Acute heart failure was induced by mechanically overloading the left ventricle through increasing the resistance of the outflow tract ^{3}H NA uptake during failure (average 22 %) was significantly lower than in the controls (average 31 %) When the failing heart was recompensated with ouabain the NA uptake was also restored to normal Ouabain alone did not have any effect on ^{3}H NA uptake The decreased NA uptake during acute heart failure was not attributed to a decreased coronary blood flow as ^{86}Rb uptake was not affected. It is suggested that decreased uptake of NA during heart failure is causally related to the hypodynamic state of the myocardium and that restoration of normal uptake after ouabain is associated with the improvement of myocardial contractile force Possible explanation of these relationships are discussed

The participation of catecholamines in the pathological state of congestive heart failure and in the therapeutic action of digitalis glycosides has long been postulated but the exact nature of such relationship remains obscure Recently a significant reduction of catecholamine content of hearts in congestive failure was demonstrated both in animals (Spann *et al* 1964 and in man (Chidsey *et al* 1963 Since it is not known whether the uptake of NA by cardiac adrenergic nerves is affected this problem was studied in the guinea pig heart lung (H L) preparation using tracer doses of tritiated NA ^{3}H NA Uptake of ^{3}H NA was determined in normal hearts hearts in acutely induced failure as well as in hearts acutely failed but subsequently recompensated with the administration of ouabain

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TABLE I Radioactive noradrenaline (^{3}H NA) and Rubidium (^{86}Rb) uptakes and cardiac noradrenaline content of guinea pig heart under various experimental conditions

Experimental condition	Number of expts	^{3}H NA uptake (%/g heart) mean±S E	^{86}Rb uptake (%/g heart) mean±S E	NA content (μg/g heart) mean±S E
Control	4	30 9±0 73	21 6±0 94	1 19±0 04
Failure	4	[1]22 1±0 53	21 8±0 66	1 18±0 07
Failure plus ouabain	4	29 9±0 50	22 1±0 82	1 22±0 03
Ouabain	4	31 9±1 50	20 9±0 45	1 23±0 05

[1] Significantly different from control, $p < 0\ 001$

Method

Guinea pigs weighing 300 to 400 g were anesthetized with urethane 1 g/kg i p The H L preparation was set up as described by Hochrein *et al* (1958) and cardiac output was measured with a Shipley Wilson rotameter The inflow of the rotameter was connected with the aortic cannula and the outflow was directed to a Starling peripheral resistance set at 600 mm Hg Right atrial pressure was measured with a Statham transducer (model 23AC) through a catheter introduced via the superior vena cava Both the cardiac output and the right atrial pressure were continuously recorded on a Grass polygraph

The

of the catheter was placed at the junction of the inferior vena cava and the right atrium Ouabain 12 μg was dissolved in 0 1 ml of saline and was given as a single injection. ^{3}H NA 5 μC, and ^{86}Rb 0 1

spectrometer Endogenous cardiac catecholamine content was determined according to the technique of Euler and Lishajko (1961) 75 % of the radioactivity present in the TCA extract was recovered in the eluate from the aluminium oxide column

Results

16 guinea pig H L preparations were used The results are summarized in Table I

Controls

In 4 H L preparations normal ^{3}H NA and ^{86}Rb uptakes were determined This averaged 30 9 and 21 6 % of the given dose per g heart) respectively (see Table I) Before the infusion of radioactive materials a myocardial competency test was carried out by observing the changes in cardiac output and right atrial pressure when venous reservoir was elevated first 3 5 cm and then 7 cm above the original level With each increment of reservoir level there was a concomitant increase in cardiac output The increase in right atrial pressure never exceeded 1 mm Hg Toward the end of the experiment

i e, 30 min after the infusion of radioactive material, cardiac output often decreased slightly, but a normal myocardial competency test could still be elicited

Acute heart failure

In 4 H L preparations acute heart failure was induced The criteria for heart failure were a decrease in the cardiac output of at least 25 %, with a rise in the right atrial pressure and failure of the heart to perform the competency test as was manifested by a further decrease in cardiac output and a sharp rise of right atrial pressure (usually to 2—3 mm Hg) when the venous reservoir level was raised Radioactive material were infused once failure became evident As can be seen from Table I, the uptake of ^{3}H NA by the failing heart was markedly and significantly lower than the control group The average uptake of ^{3}H-NA per heart during failure (22 1 %) was 28 6 % lower than that of control (30 9 %) However, ^{86}Rb uptake by the failing heart and its NA content were essentially the same as in the control group (see Table I)

Acute heart failure and ouabain

In 4 H L preparations, acute heart failure was induced as was in the previous group As soon as failure was evident (12 μg) ouabain was given Improvement of cardiac function was observed within 5 min, i e, an increase of cardiac output to or toward the control value, and a return of normal performance Radioactive materials were then infused and the experiment terminated 30 min thereafter As can be seen from Table I both ^{3}H NA and ^{86}Rb uptakes by hearts acutely failed but recompensated after ouabain were essentially the same as the uptakes of control hearts Cardiac NA content was also normal

Ouabain alone

In 4 H L preparations ouabain (12 μg) was given without the induction of heart failure This dose did not alter the cardiac output and right atrial pressure nor did it modify the myocardial competency test ^{3}H NA and ^{86}Rb uptakes as well as cardiac noradrenaline contents were not affected by ouabain administration (see Table I

Discussion

The normal guinea pig heart in the present H-L preparation was found to retain 30—35 % of the exogenously administered tracer dose of NA In hearts with acute failure however the uptake of ^{3}H NA was significantly lower This reduction was remarkably constant (an average of 29 % lower than normal) However the reduction of ^{3}H NA uptake cannot be accounted for by a decreased coronary blood flow because ^{86}Rb uptake by the heart during failure remained essentially unchanged

When acute heart failure was corrected by ouabain ^{3}H NA uptake was again found to be normal This could not be due to the effect of ouabain on the uptake of NA by the adrenergic nerves *per se* as ouabain alone had no effect on the uptake Moreover ouabain had been shown not to influence the uptake of catecholamines by the adrenergic nerve granules *in vitro* Euler and Lishajko 1965

The decreased uptake of NA by the failing heart must be causally related to the hypodynamic state of the myocardium in failure and restoration of normal uptake occurs when the myocardial contractile force is improved by ouabain

The pathological physiology of the failing heart and its modification by digitalis glycosides have been extensively studied for a review see Hajdu and Leonard [illegible] Two primary defects of the failing heart are well recognized namely a change in [illegible]

cellular ionic contents and an impairment in energy utilization and or liberation Both defects could contribute to the observed decrease in catecholamine uptake by the failing heart It is not known how changes in ionic fluxes would affect catechol amine uptake In respect to changes in energy utilization and liberation during heart failure it is well established that the former is impaired but it remains controversial whether the latter is also depressed as reduction of high energy phosphate level was found by some investigators (Szekeres and Schein 1959 Feinstein 1962 and Schwartz and Lee 1962) but not by others (Wollenberger 1947 Furchgott and deGubareff 1958 and Lee *et al* 1960) Should the energy liberation process in the myocardium be depressed catecholamine uptake could also conceivably be influenced as recent observa tions have indicated that catecholamine uptake is an active process requiring energy and that adenosine triphosphate is implicated as the energy transmitter (Hughes and Brodie 1959 Dengler *et al* 1962 Kirshner 1962 and Euler and Lishajko 1963a)

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References

CHIDSEY C A E BRAUNWALD A G MORROW and D T MASON Myocardial norepinephrine concentration in man Effects of reserpine and of congestive heart failure *New Eng J Med* 1963 *269* 653—658

DENGLER H J I A MICHAELSON H E SPIEGEL and E TITUS The uptake of labeled norepinephrine by isolated brain and other tissues of the cat *Int Neuropharmacol* 1962 *1* 23—38

EULER [illegible] chola [illegible]

EULER [illegible] uptak [illegible]

EULER [illegible] *Proc* [illegible]

FEIN[illegible] the [illegible] 333—346

FURCHGOTT R F and T DEGUBAREFF The high energy phosphate content of cardiac muscle under various experimental conditions which alter contractile strength *J Pharmacol exp Ther* 1958 *124* 203—218

HAJDU S and E LEONARD The cellular basis of cardiac glycoside action *Pharmacol Rev* 1959 *11* 173—209

HOCHREIN H H J WILKE and M STRAPAZAKIS Experimentelle Insuffizienz am Herzlungen Präparat des Meerschweinchens Naunyn-Schmiedeberg s *Arch exp Path Pharmak* 1959 *237* 533—543

HUGHES F B and B B BRODIE The mechanism of serotonin and catecholamine uptake by platelets *J Pharmacol exp Ther* 1959 *127* 96—102

KIRSHNER N Uptake of catecholamines by a particulate fraction of the adrenal medulla *J biol Chem* 1962 *237* 2311—2317

LEE K S D H YU and R BURSTEIN The effect of ouabain on the oxygen consumption, the high energy phosphates and the contractility of the cat papillary muscle *J Pharmacol exp Ther* 1960 *129* 115—122

SCHWARTZ A and K S LEE Study of heart mitochondria and glycolytic metabolism in experimentally induced cardiac failure *Circulat Res* 1962 *10* 321—33

SPANN JR J F C A CHIDSEY and E BRAUNWALD Reduction of cardiac stores of norepinephrine in experimental heart failure *Science* 1964 *145* 1439—1441

SZEKERES L and M SCHEIN Cell metabolism of the overloaded mammalian heart in situ *Cardiologica* 1959 *34* 19—27

WOLLENBERGER A On the energy rich phosphate supply of the failing heart *Amer J Physiol* 1947 *150* 733—745

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Influence of Bovine Albumin and Acids on the Respiration and Adhesiveness of Human Thrombocytes

By

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Abstract

Engel P F *Influence of bovine albumin and acids on the respiration and adhesiveness of human thrombocytes* [illegible]

HCl H_2SO_4 0 2 [illegible] ocyte consumed abo [illegible] he
10 $^{-6}$ μl hr of oxyg [illegible]
respiration decli [illegible]
6 0 after 50 hrs [illegible]
at pH 7 8—7 9 after 12 hrs, at pH 8 2—8 3 after 3 hrs, and at pH 8 6 after less than [illegible]
The adhesiveness was almost nil at pH 6 0 and about 30 % at pH 7 4—8 8

There is a close relationship between the problems of measuring the respiration of thrombocytes and leukocytes The composition of the suspension medium is important (Seelich 1959) Leukocyte suspended in serum without additives respired only shortly and with low reproducibility Esmann 1962) On the other hand, washing and suspending leukocytes in salt solutions of different kinds induced a chemical damage with aerobic production of lactic acid Warburg Gawehn and Geissler 1958 Kieler (1957) isolated leukocytes by spontaneous sedimentation of the blood, centrifuging the leukocyte containing supernatant on the top of a 30 % bovine albumin solution, and collecting the leukocytes from the bovine albumin surface With this method the bovine albumin surface stabilized the thrombocytes which were present together with the leukocytes, both with respect to respiration and adhesiveness It was the aim of this study to investigate this stabilizing effect and its relation to the pH of the medium

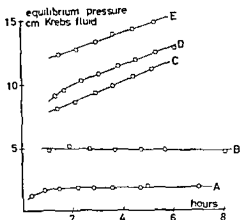


Fig 1 Equilibrium pressure as a function of time measured on divers with the following fillings A and B unbuffered plasma with and without thrombocytes C and D thrombocytes activated with, and mixed with bovine albumin E thrombocyte suspension acidified with HCl

Methods and materials

sufficient to keep the pH constant during the experiment

Calculations Equilibri

were plotted against tim

fluid corresponds to 1

was calculated The

$\Delta V = \frac{V \Delta P}{P_o}$, where V is the diver constant and P_o the barometric pressure The volume of O_2 consumed by the control was subtracted from that consumed by the specimen The difference

function Venopuncture was performed on an arm vein under stasis and with a 3d 1.2 mm cannula The blood was collected in a 7 ml polyethylene tybe (thrombotest tube con

of the stabilized blood was placed in the incubator at 37° C for 1 2 to 1 hr During this time

TABLE I Average resp rat on of human thrombocytes suspended in med a of d fferent com

Med um	No of expts	L Range	E Range	T 10⁴ Range
Unbuffered CT or CP	7	12–312	4–208	1 3(1 90
CT and CP treated w th 30% BA	6	4–639	3–549	0 99–1 72
1 µl 30% BA+3 µl CT or CP	5	4–639	3–549	0 97–1 30
CT and CP ac d fed with 0 1 N HCl	6	4–639	3–549	0 85–1 80
CT ac d fed w th 0 15 M H_2SO_4	2	591	180	1 33
1 µl 0 15 M citrate buffer in dextran +3 µl CT	15	53–1 170	17–433	0 61 1 87
1 µl 0 15 M citrate buffer n dextran +3 µl CT	8	53 837	17 7 0	0 61–1 8
1 µl 0 15 M aqueous c trate buffer +3 µl CT or CP	6	7–1 062	7 540	0 45–1 31
1 µl 0 15 M aqueous citrate buffer +3 µl CT or CP	6	7 1 062	7 540	0 45 1 31

CP Citrate stab l zed cell free plasma
CT Citrate stab l zed thrombocyte plasma suspens on
BA Aqueous bovine alb m n solut on

most blood samples sed mented so that t was poss ble to draw 500 µl f he supernatant p a a suspens on conta n ng thrombocytes leukocytes and a few red ells The cell su pens on was centrifuged for 10 to 12 m n in a m crocentr fuge (W fug) at 7 0 rp n rrespon ing to abo 33 g thus sed ment ng leukocytes and erythrocytes

Treatment of thrombocyte suspens ons w th bov e album n as perf rm ed the f l w g was 100 µl of a 30 % w w aqueous solut on of bov ne album n S atens Serum In u Copenhagen were p petted n the bot om of a centr fuge tube 500 l f thrombocy e sus pens n ere p petted on top of the album n solut on w th ut x ng the two phases The thrombocytes were sed mented on the nterphase by centr fug ng f r 1(n at 233 g and resuspe ded b s rr g the supernatant from wh ch samples ere draw f r resp ra n mea ure m ents and cell oun s Fresh s lutions of bov ne album n we e u ed e expe ment and the powder was stored well closed at 0 C

In ea h d er exper m ent thrombocyte leukocyte aln st ex lu ely s all l m phoc n and erythr cy e ounts were determ ned n the thro bocyte suspe s n us g a Le z pha e contrast ros pe f ted w th a L v Apo L 40 0 70 h obje ve Thr bocytes we e coun ed (a total f 800 1 000 cells n a Thoma s c unt ng cha ber ed tel bef re and af er f ll ng the l vers wh ch k about 15 m n If clump ng f the thr bocytes occurred ing the f ll ng the exper m en as d scarded In exper e ts w th a ified thrombocy e su pen s ons it as unne essar kee s r tly t th s procedure s e th bocy e count re na ned constant for hours

Steril tr Glassware was s red a heat ng oven at 130 C and buffers were au ocla ed fo 30 m n at 130 C Infec f the ell suspen on n sh ort term exper en s 6 8 hrs c ld be detected by O upt ke f t e e l l ver e a n ng cell free pla a In long term experi ments (1? 50 hrs ne a n rel upon a r n resp r ng c n r l n th s case n ven from the a r may pla a r le t x After su h exper ments therefore t con ents f the l em were inspected m crosc p ally and the experim ent was n lu led n f he l the c n ol and the spec men were free from a t r l gr w g and n l pl ng n org n s

¹ Maker reports that n preservat ves were a lded to the r produ t

positions and pH values

Range of CT oxygen uptake µl O/hr × 10 *	Range of CP oxygen uptake µl O_2/hr × 10 *	pH Range	Respiration average µl O_2/T/hr × 10 *	S D
0.00–2.36	−0.43–1.07	8.3–8.8	0.05	0.06
9.86–20.64	−0.87–2.76	6.3–7.1	0.92	0.13
9.70–21.40	−3.07–1.21	6.0–6.1	1.11	0.17
11.19–19.57	−0.59–1.56	6.3–6.5	1.07	0.18
12.31–14.63	—	6.0	1.03	—
7.39–23.10	—	6.3–6.5	1.10	0.14
6.45–15.88	—	5.6–5.8	0.86	0.12
4.69–14.09	−0.18–2.28	6.2–6.4	1.04	0.12
3.68–12.02	−0.28–1.47	5.5–5.7	0.82	0.15

L = Total number of leukocytes
E = Total number of erythrocytes
T = Total number of thrombocytes

Determination of the Adhesiveness of Thrombocytes. Thrombocyte suspension and cell free plasma were acidified to pH 6.0 with 0.1 N HCl. 150 µl of each were mixed with 50 µl 0.30 M tris buffer of the desired pH. Immediately after mixing, a Burker Turk counting chamber was filled with a sample of the thrombocyte mixture. The normal cover glass for the chamber was cut into two equal pieces so that each piece just covered one half of the chamber. The counting chamber was placed in a moist chamber (petri dish) and after 1 hr of sedimentation the cell count was determined (a total of 600—800 cells). Then the chamber was washed with about 150 µl of the cellfree plasma buffer mixture of the same pH. The mixture was applied at one edge of the chamber and removed from the opposite side by a piece of filter paper. The application of the mixture was performed by means of a pipette with a finely drawn tip, blown by mouth through a piece of tubing at a rate sufficient to keep the chamber completely filled during the washing. The pipette was made from a thin walled glass capillary 1.4 mm inside diameter by pulling in a flame. By this procedure thrombocytes that did not adhere to the glass surface were washed out of the chamber while thrombocytes adhering to the glass surface were not. A final counting was done on the washed chamber and the percentage of adhesion calculated.

Results

Short term experiments (6–8 hrs). Typical results obtained from short term diver experiments are seen in Fig. 1, representing equilibrium pressure as a function of time for 2 divers containing unbuffered plasma with and without thrombocytes which showed very low respirations, and for 3 divers filled with acidified (bovine albumin treated) and bovine albumin mixed thrombocyte plasma suspension which showed well defined and constant respirations during the experiments. From such curves the respirations were calculated and the results were averaged in Table I, which also contains the ranges of numbers of

leukocytes and erythrocytes contaminating the thrombocyte suspensions, the ranges of total oxygen consumption of plasma with or without thrombocytes and the ranges of pH It is seen that thrombocytes did not respire when suspended in plasma without additions where pH increased to about 9 Suspensions of thrombocytes mixed with 0 15 M citrate in dextran or water, mixed or treated with 30 % bovine albumin, or acidified with HCl or H_2SO_4 respired at levels which were not significantly different when the pH values were kept between 6 0 and 7 1 The respirations tended to attain lower values at pH 5 5—5 8

Long term experiments (50 hrs) Fig 2 shows respiration time curves from thrombocytes in media of different pH Between pH 6 0 and 7 8 one thrombocyte consumed about 10^{-8} µl O_2/hr the first 6 hrs of measurement in accordance with the results above (Table I) During the following hours the fall in respiration depended on pH, so that 1/4 to 1/2 of the respiration remained at pH 6 0 after 50 hrs The respiration diminished 10 times after 27 hrs at pH 7 4, after 12 hrs at pH 7 8, after 3 hrs at pH 8 2 and after less than 2 hrs at pH 8 6 The initial respiration at pH 8 2 was 0 6 × 10^{-8} µl O_2/T/hr, at pH 8 6 about 0 4 × 10^{-8} µl O_2/T/hr, and at pH 8 8 the initial period was too short for calculating the respiration (not included in Fig 2) All curves, except one, represents experiments with tris buffer at a final concentration of 0 075M The curve obtained from HCl-acidified thrombocytes at pH 6 0 should be compared to that with tris-acidified thrombocytes at the same pH Tris buffer did not inhibit the respiration of thrombocytes significantly at pH 6 0 Contrary to expectations the pH of HCl acidified thrombocyte suspensions at pH 6 0, kept constant during the experiment possibly because most of the CO_2 escaped during the acidification

Microscopic observations and adhesiveness Microscopic observations on thrombocyte suspensions revealed the different morphological forms as described by Tullis (1953 Floating thrombocytes usually had two or more spicules but in the case of blebbed and especially the ballooned forms, the spicules were difficult to observe since thrombocytes usually float with their cell bodies toward the observer In streaming liquid immediately after placing the cover glass, it was however, possible to see that also the ballooned and

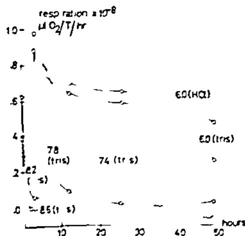


Fig 2 Respiration of human thrombocytes in cartesian divers as a function of time and pH Each point is an average of 3 determinations except the first two at pH 6 0 which were averaged from 5 determinations

TABLE II Adhesiveness of thrombocytes determined in 0 075 M tris buffered plasma thrombocyte suspensions of different pH values

Number of expts	pH	Range of adhesion %	Adhesion average %	S D
6	8 8	27–48	38	9
5	7 8	18–50	30	12
5	7 4	9–35	23	12
6	6 0	0–3	0 6	1

blebbed forms carried two or more spicules, and also to observe the process of adhesion of a thrombocyte to a glass surface The spicules adhered to the surface and tethered the cell Later the cell was surrounded by a membrane, the spicules were no longer visible and the cell cytolyzed This process was observed in unbuffered thrombocyte plasma suspensions and in buffered suspensions of pH 7 4 and higher values Immediately after placing the cover glass a fraction of the thrombocytes adhered to the glass Provided that sufficient liquid was present between the cover glass and the slide, thrombocytes treated with bovine albumin acidified to pH 6 0, or thrombocytes to which bovine albumin was added, did not adhere to the glass surface despite the presence of spicules In these cases the thrombocytes were able to float freely for a long time if the slides were protected against evaporation by sealing with vaseline On the other hand, pressing of the cover glass against the slide may be so efficient so that even "acid" thrombocytes will adhere and degenerate

A quantitative description of the adhesiveness of thrombocytes as a function of pH was possible by the method described above (Table II) At pH 6 0 the adhesiveness averaged 0 6 %, which differs significantly from the values 23—38 % measured at pH 7 4 7 8, and 8 8 The difference in adhesiveness at pH 7 4 and at pH 8 8 was not significant

Discussion

It could be inferred that the thrombocyte respiration was measured under non physiological conditions since CO_2 was absorbed However the results are used only for comparative purposes The total number of erythrocytes in the divers was considered too small to induce a stimulation of thrombocyte respiration similar to that found for leukocytes Koj Zgliczynski and Bicz 1958)

The contribution of the contaminating leukocytes to the total oxygen uptake averaged 1—2 % and was disregarded since it is small in comparison to other sources or error, the counting being the most important If 1 000 thrombocytes were counted a deviation of $0\ 94\sqrt{1,000}$ or about 6 % of the true value should be expected (Hellem 1960) The slope of the equilibrium pressure curves can be determined with an accuracy of about 2 %, so that the total error of a determination of thrombocyte respiration was about 10 %

Table I shows that the stabilization of thrombocyte respiration by bovine albumin is due to the decrease in pH, since the same effect can be produced by other acids and buffers. Similarly, the adhesion was influenced by pH so that the ability to adhere was practically zero at pH 6.0. Unbuffered thrombocyte suspensions often respired initially but lost their respiration during the first hour; this may be explained by the rise in pH caused by the absorption of CO_2. Campbell, Small and Dameshek (1956) buffered thrombocytes with phosphate buffer at pH 7, and obtained 1.0×10^{-8}—1.3×10^{-8} µl O_2/T/hr. Maupin (1954) found 0.6×10^{-8}—1.0×10^{-8} µl O_2/T/hr, and Tullis (1954) found 0.6×10^{-8}—1.8×10^{-8} µl O_2/T/hr. These values agree with our values while that of Waller *et al.* (1959) who buffered with albumin phosphate-buffer at pH 7.4 obtained the somewhat higher value 2×10^{-8} µl O_2/T/hr.

The results of this study may explain why we were unable to confirm the observations reported by Kieler (1957) that normal human leukocytes respired about ten times as much as leukemic leukocytes. Thrombocytes might have been responsible for the increased oxygen consumption of normal leukocytes because

1. Kieler's method of isolation did not exclude thrombocytes,
2. the ratio of thrombocytes to leukocytes may well be 10 times lower in leukemic blood than in normal blood,
3. bovine albumin stabilizes the respiration of thrombocytes by lowering the pH when this isolation procedure is used.

The method of Hellem (1960) for the determination of the adhesiveness of human thrombocytes required relatively much material, so we preferred a simpler method requiring only 10 µl samples and avoiding strong mechanical treatment of the cells. However, the simpler method has the drawback of being unusable for whole blood. Further, it has not yet been proved whether the washing procedure under certain circumstances may be too violent so that adhered thrombocytes could be washed away although direct microscopic observations did not indicate that this was the case. Also the condition of the surface of the chamber may influence the determination of the adhesiveness. These factors should be studied further before the method can be regarded fully suitable. In the present work it is however, only used to stress the obvious difference between the adhesion of thrombocytes at pH 6.0 and higher pH values.

As an overall conclusion the findings suggest the possibility of isolating and storing thrombocytes in such a way that also ripe thrombocytes are stabilized until their function is wanted. This may be done by 1) using citric acid as an anticoagulant instead of sodium citrate and 2) acidifying the thrombocyte suspensions immediately after isolation.

References

CAMPBELL, E. W., W. S. SMALL and W. DAMESHEK: Metabolic Activity of Human Blood Platelets. *J. Lab. clin. Med.* 1956, *47*, 835—843.

ESMANN, V.: *Carbohydrate Metabolism and Respiration in Leukocytes from Normal and Diabetic Subjects.* Universitetsforlaget, Aarhus, Denmark 1962, pp. 148.

HELLEM, A. J.: The adhesiveness of Human Blood Platelets in Vitro. *Scand. J. clin. Lab. Invest.* 1960, *12*, Suppl. 1.

HOLTER, H. and K. LINDERSTRØM-LANG: On the Cartesian Diver. *C. R. Lab. Carlsberg Sér. Chim.* 1943, *24*, 333—478.

KIELER, J.: *Cultivation of Leukemic Cells in the Cartesian Diver.* In: The leukemias, p. 215—2[illegible]. Edited by Rebuck, J. W., F. H. Bethell and R. W. Monto. Academic Press Inc. Publ. New York. 1957.

Koj, A., J. M. Zgliczynski and W. Bicz, Wpyw Erytrocytow Na Oddychanie Leukocytow Ludzkich, *Acta biochim pol* 1958 *5* 177—187

Krebs, H. A., Improved Manometric Fluid *Biochem J* 1951 *48* 240—241

Linderstrom Lang, K. and H. Holter, in Bamann and Myrback, *Methoden der Fermentforschung* Leipzig 1941 *1* 1132—1162

[illegible]

Warburg, O., K. Gawehn and A. W. Geissler, Stoffwechsel der Weissen Blutzellen, *Z Naturforschg* 1958 *13b* 515—516

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The Influence of Olfactory Stimuli upon the Activity of Secondary Neurones in the Burbot (Lota lota L.)

By

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Received 8 July 1965

Abstract

Døving K B *The influence of olfactory stimuli upon the activity of secondary neurones in the burbot (Lota lota L.)* Acta physiol scand 1966 *66* 290—299 —The nervous activity of single secondary neurones was recorded from the olfactory tract of the burbot while stimulating the olfactory epithelium with various solutions Efferent influence was eliminated by transecting the olfactory tract. About 30 per cent of the stimuli produced an increased activity in the neurones and 20 per [illegible]

The activity of the secondary neurones adapted very slowly to continuous stimula [illegible]

The morphology of the olfactory system shows great similarities throughout the Vertebrata The fibres of a large number of olfactory receptors converge in the olfactory bulb to a relatively small number of secondary neurones the axons of which transfer the sensory information to higher nervous centres In some teleosts the olfactory bulb lies close to the olfactory pit and the tract runs as a long nerve composed of two easily discerned bundles to the telencephalon (Døving and Gemne 1965) The unique anatomical feature of the olfactory system in these fishes makes it a convenient subject for electrophysiological studies of the messages conveyed in the olfactory tract

The first electrophysiological investigation of the responses to odour stimulation of the olfactory tract in fish was reported by Adrian and Ludwig in 1938 They recorded spontaneous discharges from the olfactory tract of the catfish and showed that the activity increased upon stimulation of the olfactory epithelium However, application of some substances e g anisole, inhibited the nervous activity and an intense off discharge was observed when the epithelium was washed with water Similar findings were recently reported by Boudreau (1962) in carp

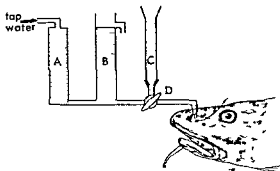


Fig 1 Schematical drawing of the stimulating apparatus and the cannula inserted into the anterior naris of the fish A, ion-exchanger, B, overflow vessel, C, cylinder for stimulating solutions, D, stop-cock

The aim of the present work was to study the activity in single afferent neurones of the olfactory tract of the burbot during natural stimulation of the olfactory epithelium The response patterns obtained from single olfactory neurones will be compared with those obtained from single units in the olfactory bulb of the frog (Døving 1964, 1965)

Methods

TABLE I The stimulating substances The different types of response are classified in the cathegories of excitatory (+) and inhibitory (—) Unaffected units have been marked (0)

Substance	Type of response			No of stimulations
	+	−	0	
Camphor	3	2	6	11
o-Chlorophenol	1	2	2	5
p-Chlorophenol	1	0	13	14
Citral	3	2	7	12
Coumarin	3	4	3	10
Diallyldisulfide	5	1	3	9
Diphenylamine	3	2	3	8
Eugenol	4	2	2	8
Fumaric acid	3	2	1	6
Geraniol	3	1	6	10
L-glutamic acid	5	1	3	9
Glutathione	5	2	2	9
8-Hydroxyquinoline	4	2	3	9
α-Ionone	5	1	3	9
β Ionone	0	1	4	5
Maleic acid	2	1	2	5
Menthol	1	1	7	9
Mesitylene	4	2	3	9
Morpholine	7	0	9	16
Pentamethylenediamine dihydrochloride	3	5	2	10
2-Phenylethanol	1	4	3	8
Pinene	2	3	9	14
Propylene glycol	2	2	7	11
Salicylaldehyde	1	3	6	10
Taurin	4	3	5	12
Thioglycollic acid	6	3	5	14
Trimethylammonium chloride	7	4	10	21
Uracil	2	2	4	8
Total	90	59	133	281
%	32	20 7	47 3	100

than at the first To measure these delays a micro-switch was operated by the stop-cock and connected to an indicator lamp at the oscilloscope screen The lamp served as a stimulus marker The time taken for the stimulating solution to enter into the olfactory pit after turning the stop-cock was [illegible] sec for the first stimulation and 9 5 sec for the second stimulation The measurements were made with a probe placed at the opening of the cannula and connected to a capacitance meter Haapanen 1962 The measurements showed that it took about 2 sec to replace the distilled water with the stimulating solution at the first stimulation and 5 sec at the second stimulation Between the applications of the different solutions the epithelium was allowed to recover for 10 min For the analysis of the data the number of impulses was

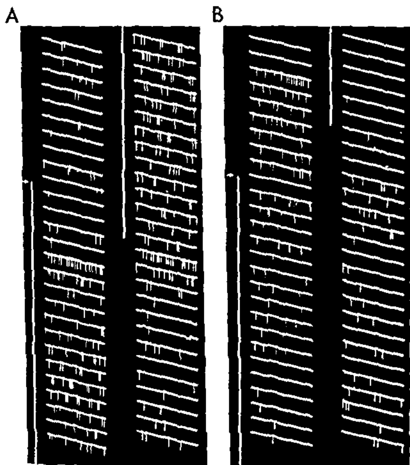


Fig 2 Recordings of the activity of a secondary olfactory neurone A excited by stimulation with pentamethylenediamin, (Unit 11/3-1), B the same unit inhibited by stimulation with 2-phenylethanol Arrows indicate start of the flow of solution Each sweep is 2 sec

counted at intervals of 2 sec (the sweep time) and the frequency was plotted against time The two curves obtained for each stimulus were superimposed on the same diagram, serving as mutual control of the responses

Results

The frequency of impulse discharges recorded from the afferent fibres in the olfactory tract varied between 1 and 9 imp sec when the epithelium was flooded with distilled water The average frequency of discharge from each unit was calculated from about six measurements, each measurement being performed by counting the number of spikes over periods of 20 sec The mean frequency of discharge ± one standard error of the mean was found to be 3 9 ± 0 3 imp sec

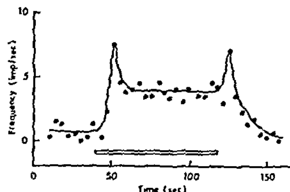


Fig 3 A typical response pattern of an afferent olfactory neurone to stimulation with menthol (Unit 11/3 1) The bars in this and the following diagrams indicate flow of stimulating solution

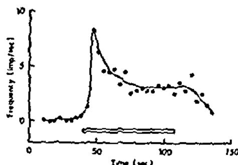


Fig 4

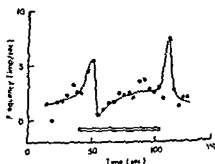


Fig 5

Fig 4 *The* response pattern evoked in an afferent olfactory neurone by stimulation with d phenylamine (Unit 11/3 1)

Fig 5 An olfactory neurone responding only to the "onset" and "*turn off*" *of the stimula* (Unit 11/3-1 *maleic* acid)

Response patterns The activity of 47 single units was recorded while stimulating with the 28 different *solutions* Some of the units were studied when applying most of the 28 substances *while some* units were tested with only one stimulating solution A total of 281 double stimulations were performed Out of the total number of stimulations about 50 per cent did not evoke any change of activity About 30 per cent of the *stimuli produced* an increased activity in the units and 20 *per cent* caused inhibition (see Table I As *mentioned in* Methods two successive stimulations were made with each solution and *in all* cases the two stimulations gave the same response pattern

Nearly *all* the solutions evoked both excitatory and inhibitory types of response Figure 2 A shows a typical record of the activity of a single afferent unit when the olfactory epithelium was stimulated with 10^{-4} M pentamethylene diamine As seen there was an increased activity when the flow of the stimulating solution is turned on and off as well as a *maintained discharge* during stimulation The response of such a unit is demonstrated graphically in Fig 3 (In each of the following frequency curves the mean frequency during 4 sec was used) As seen, there was a transient increase in the frequency followed by a steady discharge at a level of about 4/sec maintained throughout the stimulation. When the stimulus was discontinued the frequency increased again and

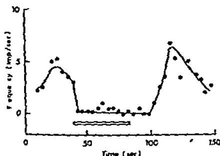


Fig 6

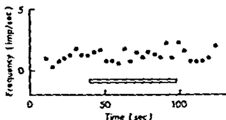


Fig 7

Fig 6 The activity of an afferent olfactory neurone inhibited by stimulation with thioglycollic acid (Unit 13/2 3)

Fig 7 An example of an afferent olfactory neurone unaffected by stimulation with propylene glycol (Unit 10/3-1)

thereafter gradually decreased to the previous level of the spontaneous activity Several stimuli gave a transient increase in activity at the onset of stimulation (Fig 4) When the stimulation was discontinued there was a gradual decrease to the normal activity level, without any off-discharge A few units were found to respond only to the onset and turn-off of the stimulation (Fig 5) In these cases, the frequency in the period between the onset and turn-off was the same as or lower than the frequency of the spontaneous activity Some units (about 6 per cent) showed a slow gradual increase in frequency during stimulation, the frequency reaching a plateau after about 30 sec. In these cases the frequency of discharge returned to the previous activity at the end of the stimulation without any off-discharge

About 20 per cent of the stimulations caused inhibition. A typical record of such a type of response is shown in Fig 2 B and also represented in the diagram in Fig 6 The spontaneous activity ceased abruptly after the onset of stimulation When the stimulation was discontinued the frequency of discharge rapidly increased to a peak value higher than the spontaneous activity These responses resemble the off responses obtained from the bulbar units in frog (Doving 1964) and from other sensory systems

As mentioned above most of the stimuli used did not have any conspicuous effect on the activity of the units An example of such a unit unaffected by stimulation with 10^{-1}M propylene glycol is given in Fig 7

Latency As seen from the response patterns illustrated in Figs 2 through 6 the latency between the onset of the stimulation and the response from the olfactory units was about 8 sec The mean value of the latencies was calculated in those cases where the increase or decrease in frequency could be measured with accuracy and was found to be 8 4 sec for the first stimulation and 12 8 sec for the second stimulation When the time lag between turning the stop-cock and the entry of the solution into the olfactory pit was subtracted, the latency due to the conduction velocities and synaptic transmission was estimated to be about 3 4 sec This long latency might partly be due to the low temperature at which the present experiments were made and partly to the rather slow onset of stimulation due to the low rate of replacement of the distilled water with stimulating solution

Adaptation As seen from Figs 2 A, 3 and 4, the activity of the secondary neurones adapts very slowly to continuous stimulation The difference between the peak frequency and the maintained discharge varied from one response to another and was greatest for "on-off" response patterns like the one demonstrated in Fig 5 an effect most likely due to inhibitory interactions between the bulbar units In the present study different concentrations of one odour were not applied and the adaptation rates at different stimulus strength were not studied The slow rate of adaptation of the secondary neurones in burbot confirms the results obtained by Adrian and Ludwig (1938) on catfish Further evidence for the slow rate of adaptation has been reported at other levels of the olfactory system in other animals Thus Adrian (1950 1956) demonstrated that the mitral cells of the olfactory bulb in rabbit gave a distinct discharge in response to each inspiration which did not change over periods up to one hour Observation of the electro-olfactogram (EOG) on frog and rabbit give evidence that the olfactory receptors adapt slowly (Ottoson 1956 1959 a b) Experiments made by Shibuya (1960) indicate that the receptor organ of fish also adapts slowly Hence, the slow rate of adaptation in the olfactory receptors is faithfully reproduced in the discharges of the secondary neurones

Discrimination Out of a total number of 30 units investigated for two or more different stimuli 6 units gave the same type of response to all solutions *i e* they did not show any specific reaction Half of the total number of units were found to give different types of response to the different olfactory stimuli One unit was found to be excited by fumaric acid and inhibited by maleic acid Units giving different types of response to the α and β forms of ionone were also found but none of the units gave different types of response to the *ortho* and *para* form of chlorophenol Most of the substances evoked both excitatory and inhibitory types of response the only exception being p-chlorophenol and morpholine which failed to inhibit any of the units studied and β ionone which did not excite any unit From Table I it can be seen that the chemicals least effective in evoking excitatory or inhibitory types of response were p-chlorophenol β ionone and menthol. The most effective chemicals were fumaric acid pentamethylene diamine glutathione eugenol and coumarin

To obtain information about the discriminatory power of the olfactory system a comparison was made of the number of similar and different types of response The method was applied in a previous study on the frog (Döving 1965) which dealt with the types of response associated with combinations of 5 odours taken 2 3 or 4 at a time In the present study the different combinations of stimuli were taken two at a time and the number of similar and different types of response was counted If all the substances have similar olfactory properties or if the discriminatory power of the system is low one would expect to find a larger proportion of similar than of different types of response Out of the total number of pairs counted in the present study 1098 gave similar and 1028 gave different types of response to the two stimuli in the pairs Hence about 48 per cent of the total number of pairs gave different types of response This value is almost the same as that obtained in the previous study Döving 1965 The results indicate that the discriminatory power of the olfactory system is fairly well developed in the burbot

Six of the substances used in the present study viz maleic acid fumaric acid glutathione taurin and uracil are not perceived as odours by humans because of the non volatility of these compounds However non volatile substances may evoke a sensation of smell when introduced to the olfactory epithelium in solution Backman 1917) or

when injected intravenously (for review see Ottoson 1963) The six non volatile substances used in the present study influenced the activity of the secondary olfactory neurones more frequently (65 per cent) than did the other ones (50 per cent)

Discussion

The burbot is a bottom dweller with well developed olfactory organs (Wunder 1927 Teichmann 1954 Pfeiffer 1965) and rather poorly developed eyes (Engstrom 1961) In Lake Malaren (near Stockholm) burbots with infected or degenerated eyes but otherwise in good condition are frequently found These findings and the indication that the burbot is mainly active during the night and the dawn (Wikgren 1955 indicate that vision is of minor importance for survival The burbot feeds on fish fish refuse and bottom dwelling invertebrates (Sasserson 1964) Especially in finding the fish refuse the sense of smell must be of greater importance than *e g*, the mechanical sense organs

The recordings in the present experiments were made from the transected olfactory tract which implies that the activity recorded originated from afferent fibres and was uninfluenced by efferent impulses On the basis of the anatomical features of the olfactory system it seems reasonable to assume that these fibres arise from secondary neurones separated by only one synapse from the olfactory receptors (Sheldon 1921 Holmgren 1920) In the tract of the burbot there are non myelinated fibres about 0 5 μ thick and myelinated fibres 0 5—7 μ in diameter (Doving and Gemne 1963 1965) Single unit activity was recorded from both bundles of the tract and presumably from the myelinated axons

The response patterns resemble those usually found in the neurones of the second or higher order in other sensory systems and confirm the findings of Adrian and Ludwig (1938) on the olfactory tract of catfish The response patterns are similar to the ones observed from the bulbar units of the frog (Doving 1964) However the gradually increasing response observed in about six per cent of the present stimulations was not observed in the latter study A possible explanation for the occurrence of on-off responses found in the present work might be the high concentrations of stimulating solutions In spite of the comparatively high concentrations used about half the number of stimuli did not affect the units and a small proportion of the units were inhibited This finding is in striking contrast to the observations in the frog where most of the investigated units are inhibited by natural stimuli and only about 10 per cent are unaffected (Doving 1964 1965) The observation that fewer units were affected in the burbot than in the frog may reflect the less complex organization of the bulbar units in fish (*cf* Ramon y Cajal 1894 van Gehuchten 1894 Sheldon 1912 Holmgren 1920 Ariens Kappers Huber and Crosby 1936 Allison 1953) and indicates that the information processing is not developed to the same extent as in frog

In the present study none of the chemicals used failed to influence all the units examined Six of the substances (*viz* fumaric acid maleic acid glutamic acid glutathione taurin and uracil) are not perceived as odours by humans when applied in a natural way presumably due to the fact that they are non volatile However these non-odourous substances more frequently evoked responses in the burbot than did the 22 other substances It has been demonstrated that many of these six substances evoke specific responses in various aquatic animals Thus, glutamic acid elicits a feeding response in *Bathytoma nimator* (Steven 1959) and evokes nervous activity from the supra-oesophageal ganglion of *Buccinum undatum* when applied to the *osphradium* (Bailey and Laverack

1963) Glutathione has been shown to be a specific agent in the activation of the feeding reflex in *Hydra* (Loomis 1955, Cliffe and Waley 1958 Lenhoff and Bovaird 1961)

In previous studies on frog (Döving 1964, 1965) the activity of bulbar units was studied when stimulating the olfactory epithelium with different odours In the latter study a statistical method was applied to estimate the similarity of the stimulating properties of the odours This kind of analysis could not be performed on the present material due to the small number of units investigated However, the present study on burbot indicates that the discriminatory power of the olfactory system is fairly well developed as indicated by the fact that some units were differentially effected by isomeric molecules Furthermore, in about half of the combinations of two olfactory stimuli the units showed different types of response to the two stimuli, which is almost the same value as that found in frog (Döving 1965)

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References

ADRIAN E. D The electrical activity of the mammalian olfactory bulb *Electroenceph. clin Neurophysiol* 1950 *2* 377—388

ADRIAN E D The action of the mammalian olfactory organ *J Laryng* 1956 *70* 1—14

ADRIAN E D and C LUDWIG Nervous discharge from the olfactory organs of fish. *J Physiol* (Lond) 1938 *94* 441—460

ALLISON A C The morphology of the olfactory system in the vertebrates *Biol Rev* 1953 *28* 195—244

ARIËNS KAPPERS C U G C HUBER and E C. CROSBY The comparative anatomy of the nervous system of vertebrates including man. *Macmillan New York* 1936

BACKMAN E L Experimentella undersökningar över luktsinnets fysiologi *Upsala Läk.-Fören. Förh* 1917 *22* 319—464

BAILEY D F ... of a gastropod ... 12

BOUDREAU J C ... 272 278

CLIFFE E E and S G WALEY Effects of analogous of glutathione on the feeding reaction of *Hydra Nature* (Lond 1958 *182* 804—805

DÖVING K ... and single uni... ub.

DÖVING K ... *Rev Laryng* Bordeaux) 1965 In press

DÖVING K B and G GEMNE Fiber spectra and compound action potential of the olfactory tract in fish (*Lota lota* L) *Acta physiol scand* 1963 *59* Suppl 213

DÖVING K B and G GEMNE Electrophysiological and histological properties of the olfactory tract of the burbot *Lota lota* L *J Neurophysiol* 1965 *28* 139—153

DÖVING K B and G GEMNE An electrophysiological study of the efferent olfactory system in the burbot 1966 To be publ

ENGSTRÖM K Cone types and cone arrangement in the retina of some gadids *Acta Zool* (Stockh) 1961 *42* 227—243

GEHUCHTEN A VAN Contribution à l etude du systeme nerveux des téléostéens *Cellule* 1894 *10* 255—295

HAAPANEN L E A high-sensitivity capacitance meter *Electronic Eng* 1962 *34* 183—185

HOLMGREN N Zur Anatomie und Histologie des Vorder und Zwischenhirns der Knochenfische *Acta Zool* (Stockh) 1920 *1* 137—315

LENHOFF H M and J BOVAIRD Action of glutamic acid and glutathione analogous on the *Hydra* glutathione receptor *Nature* (Lond) 1961 *189* 486—487

LOOMIS W F, Glutathione control of the specific feeding reactions of *Hydra* *Ann N Y Acad Sci* 1955 *66* 211
OTTOSON D Analysis of the electrical activity of the olfactory epithelium *Acta physiol scand* 1956 *35* Suppl 122
OTTOSON D Studies on slow potentials in the rabbit's olfactory bulb and nasal mucosa *Acta physiol scand* 1959 a *47* 136—148
[illegible] ...ust and olfactory
[illegible] *Re* 1963 *15*

PFEIFFER W The size of the olfactory organ in the burbot *Lota lota* (L.) *Canad J Zool* 1965 *43* 223—224
RAMON Y CAJAL, S D Notas preventivas sobre la estructura del encefalo de los teleosteos *An Soc esp Hist nat* 1894 *23* 93—101
SASSERSON I Pers comm 1964
SHELDON R E The olfactory tracts and centers in teleosts *J comp Neurol* 1912 *22* 177 339
SHIBUYA, T The electrical responses of the olfactory epithelium of some fishes *Jap J Physiol* 1960 *10* 317—326
STEVEN D M, Studies on the shoaling behavior of fish *J exp Biol* 1959 *36* 261—280
TEICHMANN H Vergleichende Untersuchungen an der Nase der Fische *Z Morph Ökol Tiere* 1954 *43* 171—212
TEICHMANN H, Die Chemorezeption der Fische *Ergebn Biol* 1962 *25* 177—205
WIKGREN B J Daily activity pattern of the burbot *Memor Soc Fauna Flor fenn* 1956 *31* 91—97
WUNDER W, Sinnesphysiologische Untersuchungen über die Nahrungsaufnahme bei verschiedenen Knochenfischarten *Z vergl Physiol* 1927 *6* 67—98.

Acta physiol. scand. 1966. 66. 300—306

From the Institute for Medical Biochemistry, University of Oslo, Norway

Insulin Antagonistic Effect of Human Plasma Albumin on Protein Synthesis in vitro and on Glycogen Synthesis in vivo in the Rat Diaphragm Muscle

By

JAK JERVELL

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Abstract

Jervell, J. *Insulin antagonistic effect of human plasma albumin on protein synthesis in vitro and on glycogen synthesis in vivo in the rat diaphragm muscle.* Acta physiol. scand. 1966. 66. 300—306. — The earlier finding of an antagonistic effect of human plasma albumin on the insulin-stimulated glucose uptake of the cut rat diaphragm in vitro has been confirmed. In the intact rat hemidiaphragm *in vitro* 1 mU. of insulin per ml caused a marked increase (40—70 per cent) of the incorporation of ^{14}C-labelled phenylalanine into protein. Adding 5 per cent albumin caused a significant reduction of this insulin effect. Intraperitoneal injection of 1mU. of insulin caused a marked increase in glycogen content and ^{14}C-labelled glucose incorporation into glycogen in the diaphragm muscle *in vivo*. This effect was antagonized by the simultaneous injection of 50—150 mg of "antagonistic" albumin; control injections of "non antagonistic" albumin had no such effect. Increasing the insulin dose 10-fold abolished the antagonistic effect of the albumin. The results support the assumption that the "synalbumin" antagonist is a physiological antagonist of insulin action.

The effect of insulin on the glucose upake of rat diaphragm in vitro is reduced by the addition of 3.5—5.5 per cent normal human plasma albumin to the incubation medium. (Vallance-Owen, Dennes and Campbell 1958, Lowy, Blanshard and Phear 1961.) Preliminary investigations of the effect of the albumin on other insulin-sensitive reactions in the diaphragm have been reported by Jervell (1963) and Buse and Buse (1964).

In the present study the effect of the antagonistic albumin on the insulin stimulation of protein synthesis was investigated, using the intact diaphragm preparation. An in vivo effect of the albumin was sought and found using intraperitoneal injection of albumin and insulin and testing the effect on diaphragm glycogen.

TABLE I The insulin antagonistic effect of various albumin preparations on glucose uptake of the cut hemidiaphragm

	Glucose uptake (mg/g dry tissue per 90 min)				
Albumin no	Buffer alone	5 % albumin	1 mU insulin per ml	Insulin +albumin	Significance of difference between uptakes in presence of insulin ± albumin
I	28 5±1 9 (6)	28 9±1 0 (5)	51 2±1 3 (6)	50 3±2 2 (6)	No difference
III	26 2±1 6 (6)	25 3±1 3 (6)	52 8±2 2 (6)	40 7±1 7 (6)	p< 0 005
IV	25 6±1 9 (3)	24 2±1 4 (3)	50 2±1 6 (9)	43 0±1 8 (9)	p< 0 01
V	19 5±0 5 (3)	31 3±0 1 (3)	41 8±0 1 (3)	34 3±0 8 (3)	p< 0 005
VI	24 6±1 3 (6)	34 3±1 3 (6)	47 6±1 2 (6)	41 2±1 0 (6)	p< 0 005
VIII	27 0±1 1 (3)	26 8±0 1 (3)	56 7±2 5 (3)	47 7±0 2 (3)	p< 0 025
IX	27 3±1 2 (6)	27 9±1 8 (6)	55 5±2 0 (6)	44 3±2 0 (6)	p< 0 005

Each cut hemidiaphragm was incubated for 90 min at 37 C in 1 ml Krebs-Ringer bicarbonate buffer with 3 mg glucose per ml and additions of insulin and albumin as indicated Albumin I is the non-antagonistic KABI albumin, albumins III—IX were isolated from plasma of various normal subjects

Standard error of mean and number of observations are given with each figure

Methods

sodium hydroxide before freeze-drying The albumin preparations thus treated did not alter the pH of the Krebs-Ringer incubation buffer Commercial human albumin from AB Kabi was treated on ...

95 % O_2.

The intact hemidiaphragm was used to study protein and glycogen synthesis in vitro In the intact diaphragm the insertions to the rib cage are kept intact thereby keeping the number

cut muscle fibers at a minimum (Kipnis and Cori 1957) By dividing this preparation along the [illegible]

blotted on filter paper and weighed For determination of glycogen they were placed on 0.5 ml 30 per cent potassium hydroxide, for isolation of protein in 10 per cent trichloroacetic acid

[illegible]

were analysed separately thus the mean values are based on 8 estimations

[illegible]

reagent was then added directly to the neutralized hydrolysate The amount of glycogen found by this method was 93 5 per cent of that found by the anthrone method (S E M = 1 9 per cent 16 observations)

The radioactivity of the glycogen was determined by applying 2 aliquots of 200 μl on plastic planchets, and after drying counting them in a window less methane gas flow counter (Frieseke Hoepfner) equipped with a cathode grid No corrections for self-absorption were made on these very thin samples Each planchet was counted twice

The proteins were extracted as described by Manchester and Young (1958), the homogenizations however were carried out in all an glass Potter Elvehjelm homogenizer The dry protein was weighed and dissolved in one or two ml of concentrated formic acid Duplicate aliquots of 100 μl were applied on plastic planchets dried and counted as described for glycogen. No correlation for self absorption was made, as the amount of protein was almost the same in all diaphragms

Materials

Bovine insulin 10 crystalized lot no 05462 was obtained from Novo Industri A S the activity was assumed to be 23 6 units per mg ^{14}C-(U)-D-glucose 3 9 mC per mM and ^{14}C-(U)-L-phenylalanine 9 1 mC per mM was obtained from the Radiochemical Centre, Amersham, England. Standard high purity chemical and biochemical reagents were used throughout.

Results

Table I shows the insulin-antagonistic activity of the albumin used in this study All except the commercial albumin which had been treated in a column of acetylated cellulose reduced the effect of 1 ml of insulin per ml on the glucose uptake of the cut [illegible] diaphragm. Preparation I will be called non-antagonistic, while the other [illegible]

[illegible] lalanine into protein in [illegible]

[illegible] paration 1 ml of insulin

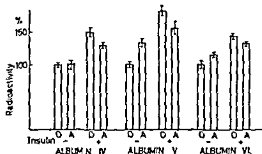


Fig 1 The effect of human albumin on the incorporation of ^{14}C-labelled phenylalanine into protein in the intact hemidiaphragm

4 intact hemidiaphragms from 20-hour fasted rats were incubated for 2 hours at 37° C in 10 ml Krebs-Ringer bicarbonate buffer 2 μC ^{14}C-phenylalanine was added to each incubation flask and 5 per cent albumin and 1 mU of insulin per ml were added as indicated (0 = no albumin A = 5 per cent albumin)

The columns indicate relative radioactivity in tissue protein (100 per cent = incorporation under basal conditions) Each column represents the mean of results from 8 to 16 hemidiaphragms The reduction of incorporation in the presence of insulin caused by the albumin (insulin antagonistic effect) is significant in all experiments ($p < 0.05$) The roman numerals refer to the albumin preparation used see the table for their antagonistic effect on the glucose uptake of the cut hemidiaphragm

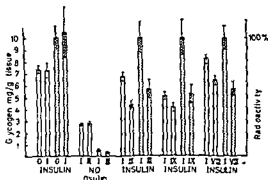


Fig 2 The insulin antagonistic effect of human plasma albumin on glycogen synthesis in the rat diaphragm in vivo

2 ml Krebs Ringer bicarbonate buffer with 2 μC ^{14}C-glucose per ml injected i p in rats fasted for 20 hrs 100 mg albumin and 1 mU insulin added to the injection as indicated Open columns glycogen content in diaphragms after 2 hrs, hatched columns glycogen radioactivity (cpm per g tissue) incorporation with insulin and non antagonistic albumin = 100 per cent Each column is mean of 8 determinations on hemidiaphragms from 4 rats with S E M The reduction of insulin effect caused by albumins III VIII and XI is significant ($p < 0.01$ for III and VIII $p < 0.05$ for IX) Roman numerals refer to the albumin preparation (see Table I)

per ml had little effect causing only 20 per cent stimulation above the basal incorporation By increasing the insulin concentration to 100 mU per ml the insulin effect was increased to around 70 per cent above basal At this high insulin concentration however, the antagonistic effect of albumin was low However using the intact hemidiaphragm, 1 mU of insulin per ml stimulated the incorporation of radioactive phenylalanine into proteins by 40 to 70 per cent above the basal incorporation (Fig 1) Three albumin preparations were tested and all reduced the insulin effect Those albumins which increased the basal glucose uptake also increased the incorporation of phenylalanine into protein in the absence of insulin [1]

[1] Preliminary investigations have shown that some albumin preparations contain significant amounts of immunologically active insulin this might explain the stimulatory effect of the albumin on the incorporation in the absence of added insulin

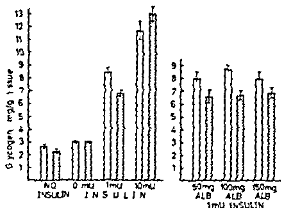


Fig 3 The insulin antagonistic effect of human plasma albumin in vivo Effect of varying the amount of insulin and albumin injected

2 ml Krebs Ringer bicarbonate buffer injected i p in rats fasted for 20 hrs Albumin and insulin added as indicated Open columns glycogen content in diaphragms after 2 hrs with non antagonistic KABI albumin (I) hatched columns with antagonistic albumin (IX see Table I) Each column mean of 8 determinations on hemidiaphragms from 4 rats with S E M

The intact hemidiaphragm preparation was also used to study the effect of antagonistic albumin on glycogen synthesis Unequivocal results however, were hard to obtain and the experiments were abandoned in preference to i p injections of insulin and albumin The results of these experiments are shown in Fig 2 It is seen that the injection of antagonistic albumin markedly reduced the effect of insulin on glycogen synthesis in the rat diaphragm measured both by chemical analysis or by incorporation of ^{14}C-labelled glucose The non antagonistic Kabi albumin however did not have this effect Antagonistic albumin did not affect the diaphragm glycogen when no insulin was injected Similarly the effect of insulin was the same when no albumin was injected as when the non antagonistic albumin was used

The effect of increasing the amount of insulin injected is shown in Fig 3 left half. In these experiments the amount of albumin injected was kept constant (100 mg) When the insulin dose was 0 1 mU there was little or no insulin effect and no insulin-antagonistic effect could be demonstrated When 1 mU was injected the same antagonistic effect as shown earlier was found However, when 10 mU of insulin were given, the antagonistic effect of the albumin was abolished

On the other hand increasing the amount of albumin injected from 50 to 150 mg appears to make no difference (Fig 3 right half) This may be explained by supposing that the albumin is drained through the diaphragm at a rate which is relatively independent of the amount of protein injected into the peritoneal cavity

It was observed that the effect of insulin upon glycogen synthesis in vivo after i p injection was on the average 25 per cent (standard deviation 11 per cent) higher in the right than in the left hemidiaphragm while there was no difference in glycogen content between the two sides when no insulin had been injected It is known that a major part of i p injected protein is drained through the diaphragm (Abdov Reinhardt and Tarver 1952) and the difference between the two sides can best be explained by supposing that the drainage through the right hemidiaphragm is greater than through the left. This has been found in the dog by Courtice and Steinbeck (1979)

Discussion

With few exceptions (Keen 1963 Cameron Keen and Menzinger 1964) there now seems to be general agreement that human plasma albumin will reduce the effect

of insulin on the glucose uptake of the cut rat hemidiaphragm in vitro (Vallance-Owen *et al* 1958 Lowy *et al* 1961, Jervell 1963 Alp and Recant 1964) The effect is relatively independent of the method used for the isolation of the albumin and also of the albumin s content of free fatty acids and its calcium and magnesium binding property The effect is still present when the decrease in the pH of the incubation medium caused by the albumin has been corrected (Jervell 1965) The insulin antagonist associated with human plasma albumin has been called the synalbumin antagonist (Kipnis and Stein 1964)

It is known that insulin stimulates protein synthesis and independently the transport of amino acids from the extracellular to the intracellular space This was first shown for the unnatural nonutilized α amino-iso-butyric acid, but has also been demonstrated for several natural amino acids (Wool 1964) It can be demonstrated for most or all amino acids (including phenylalanine which was used in the present study) when protein synthesis is inhibited by puromycin (Castles and Wool 1964)

The antagonistic effect of the albumin on the insulin stimulation of protein synthesis must be considered an effect independent of decreased glucose uptake since the experiments were performed in the absence of glucose after the diaphragm had been soaked for 20—30 min in glucose free buffer The effect of the synalbumin antagonist both on protein synthesis and glucose uptake indicates that it acts at some early stage common

The effect of the synalbumin antagonist on various insulin sensitive reactions in the rat diaphragm has been reported earlier by Jervell (1963) and Buse and Buse (1964) Jervell, however, did not at that time adjust the pH of the albumin containing media, these preliminary results are therefore not completely valid Buse and Buse in their studies used Cohn fractionated albumin which lost its antagonistic activity upon dialysis Their antagonistic activity is therefore due to some dialysable contaminant of the albumin and not identical with the synalbumin antagonist which is not lost upon dialysis

For valid reasons doubts have been raised about the physiological importance of the finding of insulin antagonism on the highly unphysiological cut diaphragm in vitro (Kipnis and Stein 1964) The demonstration of an effect of the antagonist both on the intact diaphragm and after intraperitoneal injection is therefore encouraging and should make it more attractive to examine the physiological and pathological roles of the antagonist more closely

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References

Abdou J A W Reinhardt and H Tarver Plasma protein III The equilibrium between plasma and lymph protein *J biol Chem* 1952 *194* 15—23

Alp H and L. Recant Effect of the insulin inhibitory albumin fraction from normal and diabetic subjects on adipose tissue *Metabolism* 1964 *13* 609—619

BUSE, M G and J BUSE Insulin antagonism associated with albumin Excerpta Medica 1964 Int Congr Ser 74 169 Abstr Fifth Congr Int Diab Fed

CAMERON J S H KEEN and G MENZINGER Insulin activity of normal plasma and plasma acid ethanol extracts *Lancet* 1964 *I* 74—77

CARROL, N V, R W LONGLEY and J H ROE The determination of glycogen in liver and [illegible]

C[illegible]

E[illegible]

HIGGINS G M and A S GRAHAM Lymphatic drainage from the peritoneal c[illegible] dog *Arch Surg* 1929 *19* 453—465

HUGGETT A ST G and D A N[illegible] [illegible]e in determ[illegible]

JERVELL [illegible] up-take [illegible] *J* 1963

JERVELL, [illegible] an plasma albumin on the insulin stimulated glucose [illegible] isolated rat diaphragm *Acta physiol scand* 1965 *65* 33—44

KEEN H Properties of human "albumin" A metabolic study of albumin extracts from normal and diabetic plasma *Diabetes* 1963 *12* 406—413

KIPNIS D M and C F CORI Studies on tissue permeability III The effect of insulin on pentose uptake by the diaphragm *J biol Chem* 1957 *224* 681—693

KIPNIS D M and M F STEIN Insulin antagonism Fundamantal considerations Ciba Foundation Colloquia on Endocrinology *The Aetiology of diabetes mellitus and its complications* 1964 *15* 156—184

KONO T and S P COLOWICK Stereospecific sugar transport caused by uncouplers and SH inhibitors in rat diaphragm *Arch Biochem* 1961 *93* 514—519

LOWY C G BLANSHARD and D PHEAR Antagonism of insulin by albumin *Lancet* 1961 *I* 802—804

LUKENS F D W Insulin and protein metabolism *Diabetes* 1964 *13* 451—461

MANCHESTER K L and F G YOUNG The effect of insulin on incorporation of amino acids into protein of normal rat diaphragm in vitro *Biochem J* 1958 *70* 353—358

MICHAEL S E The isolation of albumin from blood serum or plasma by means of organic solvents *Biochem J* 1962 *82* 212—218

RAFAELSEN O J Glycogen content of rat diaphragm after intraperitoneal injection of insulin and other hormones *Acta physiol scand* 1964 *61* 314 322

RAFAELSEN O J V LAURIS and A E RENOLD Localized intraperitoneal action of insulin on rat diaphragm and epididymal adipose tissue in vivo *Diabetes* 1965 *14* 19 26

UMBREIT W W R H BURRIS and J F STAUFFER *Manometeric techniques* 1957 *3* 149 Burgess Publ Co Minneapolis 15 Minn

VALLANCE-OWEN J E DENNES and P N CAMPBELL Insulin antagonism in plasma of diabetic patients and normal subjects *Lancet* 1958 *II* 336—338

WALAAS O and E WALAAS Effect of epinephrine on rat diaphragm *J biol Chem* 1950 *187* 769—776

WOOL J G Effect of insulin on accumulation of radioactivity from amino-acid by isolated intact rat diaphragm *Nature* (Lond) 1964 *202* 196—197

Acta physiol. scand. 1966 66, 307—315

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Effect of Puromycin and Puromycin Analogues on Glycogen Synthesis in the Isolated Rat Diaphragm[1]

By

ODDMUND SOVIK

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Abstract

SOVIK, O *Effect of puromycin and puromycin analogues on glycogen synthesis in the isolated rat diaphragm* Acta physiol scand 1966 66 307—315 — In a previous work from this laboratory it was reported that puromycin specifically depressed the insulin induced glycogen synthesis in isolated rat diaphragm In the present work the effect of puromycin on glycogen synthesis has been studied under modified experimental conditions When glucose as a substrate was excluded from the incubation medium puromycin depressed the incorporation of ^{14}C-glucose into glycogen in absence of added insulin To study this effect of puromycin experiments with puromycin analogues were undertaken At a concentration of 270 μg/ml puromycin inhibited protein synthesis by more than 90 % Equimolar concentrations of puromycin aminonucleoside and 6-dimethylaminopurine caused only slight inhibition of protein synthesis but depressed the incorporation of ^{14}C-glucose into glycogen to the same degree as puromycin The results suggest that puromycin influences the synthesis of muscle glycogen by a mechanism not related to the inhibitory effect of puromycin on protein synthesis The possibility that puromycin might interfere with the activity of glycogen synthetase is discussed

Puromycin[2] is known as a specific inhibitor of protein synthesis in mammalian cells (Yarmolinsky and de la Haba 1959) It acts probably by combining with polypeptides in the course of protein synthesis and thereby causes release of incomplete peptide chains (Nathans 1964) However, evidence has been obtained that puromycin might have effects different from the effect on protein synthesis (Hofert *et al* 1962, Hofert and Boutwell 1963 Korner and Raben 1964) The *in vitro* experiments presented here lend support to this view

During the course of studies on the relationship between protein synthesis and the metabolic effects of insulin it was observed that puromycin caused a depression of the insulin induced glycogen synthesis in isolated rat diaphragm (Sovik and Walaas 1964,

[1] A preliminary report of these results was presented at the 2nd Meeting of the Federation of European Biochemical Societies Vienna 1965

[2] 6-dimethylamino-9 (3 p-methoxy L-phenylalanylamino-3 -deoxy β-D-ribofuranosyl) purine

Sovik 1965) The fact that puromycin in these experiments had no effect on glycogen synthesis in the absence of added insulin suggested that puromycin specifically inhibited the effect of insulin, either by inhibiting an insulin-induced protein synthesis, or by interfering with an insulin-stimulated reaction not involving protein synthesis In order to study these two possibilities, experiments with puromycin analogues were undertaken The puromycin analogues PAN[1] and 6-DAP, which have only weak effects on hepatic protein synthesis, have been shown to cause a depression of liver glycogen even more pronounced than puromycin (Hofert and Boutwell 1963)

Materials and methods

Male and female rats, 100—160 g, from the strain at the institute, were kept on standard diet Before the diaphragms were removed, the animals were fasted 18—24 hrs, then killed by a blow on the head and decapitated

Materials

Puromycin dihydrochloride and PAN, purchased from Nutrition Biochem Corp, and 6 DAP

analytical grade

Incubation techniques

weighed and subjected (puromycin, PAN or ontrols Krebs Ringer phosphate buffer, pH 7.2—7.4, gassed with O_2 was used on was performed for 15—30 min at room temperature (20—22° C) Thereupon ^{14}C labelled compounds, and in some experiments unlabelled glucose, were added to the appropriate concentrations, the vessels stoppered, gassed with O_2 for 5—10 min and incubated in a Dubnoff shaker (100 cycles/min) After incubation the diaphragms were blotted on filter paper and subjected to tissue extraction and analytical procedures

Analytical procedures

Glycogen was isolated according to Walaas and Walaas (1950) and determined by the anthrone method of Caroll *et al* (1956) ^{14}C labelled glycogen was dissolved in water or 0 1 N H_2SO_4 and samples of 0 1—0 2 ml were applied on plates dried and counted in a windowless methane flow counter (Frieseke & Hoepfner) at infinite thinness (less than 1 mg/cm²), ^{14}C-labelled ... acid

Results

Glucose uptake in Presence of Puromycin

It has previously been shown (Eboué-Bonis *et al* 1963 Sovik 1965) that puromycin in doses of 270—500 $\mu g/ml$ does not affect glucose uptake in isolated rat diaphragm

[1] Abbreviations
PAN: Puromycin aminonucleoside 6-DAP 6-dimethylaminopurine

TABLE I Effect of puromycin on the incorporation of ^{14}C-glucose into glycogen Paired hemidiaphragms were preincubated 30 min with or without puromycin (270 μg/ml), and then incubated 10 min in the same media, to which had been added ^{14}C-glucose (0 5 μC/ml) and in some experiments unlabelled glucose (2 mg/ml) The amount of tracer glucose added was about 25 μg/ml Glycogen was isolated and determined as described under "analytical procedures"

Addition to incubation medium	Number of experiments	Rate of incorporation of ^{14}C-glucose into glycogen	Amount of glycogen	Specific activity of glycogen
		counts/min/100 mg wet tissue[1]	μg/100 mg wet tissue[1]	counts/min/μg[1]
Tracer glucose				
No puromycin	6	[2]4,489 ± 529	82 ± 9	[3]56 ± 4
Puromycin added	6	[2]2,851 ± 328	85 ± 12	[3]35 ± 3
Tracer glucose + unlabelled glucose				
No puromycin	6	1,987 ± 240	157 ± 17	[4]14 ± 2
Puromycin added	6	1,834 ± 279	193 ± 19	[4]10 ± 2

[1] ± standard error of the mean [2] $p < 0\,005$ [3] $p < 0\,001$ [4] $p < 0\,02$

The important question whether puromycin might inhibit the uptake of glucose by the tissue was re-examined by the following procedure Paired hemidiaphragms were preincubated 30 min with or without puromycin (270 μg/ml) and incubated 10 min with ^{14}C-glucose (0 5 μC/ml) Aliquots from the incubation media were subjected to paper chromatography in butanol acetic acid H_2O (4 1 5) for 20 hrs The radioactivity of the glucose peak was counted in a windowless methane flow counter (Frieseke & Hoepfner) No significant difference in the uptake of ^{14}C-glucose in the absence and presence of puromycin could be detected by this procedure

Effect of Puromycin on Glycogen Synthesis

Table I shows the effect of puromycin in experiments with a brief incubation time In some [illegible]

the amount of glycogen and in contrast to earlier findings (Sovik 1965) it caused a slight decrease in the glycogen specific activity At the lower glucose concentration puromycin caused a marked decrease of the incorporation of ^{14}C-glucose into glycogen The incorporation per unit tissue weight and the specific activity were equally depressed No change was observed in the amount of glycogen

The possibility that puromycin might reduce the specific activity of glycogen by an isotope dilution effect was investigated Carlin and Hechter (1963) observed that the insulin-induced incorporation of ^{14}C-glucose into glycogen was somewhat reduced by puromycin, and proposed that as a result of puromycin treatment endogenous amino

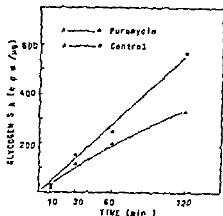


Fig 1

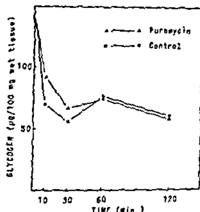


Fig 2

Fig 1 Time course for the effect of puromycin on the specific activity of glycogen (Glycogen S A) Paired

without puromycin (270

ved after the time periods

"analytical procedures"

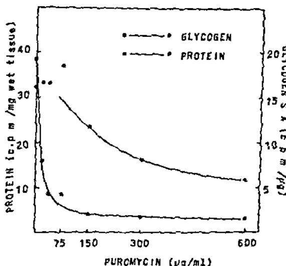


Fig 3 *Puromycin dose response curves for ^{14}C-labelling of glycogen and protein* The values for glycogen and protein are from different experiments When glycogen labelling was studied hemidiaphragms were preincubated 30 min with puromycin and then incubated 10 min after addition of ^{14}C-glucose (0 8 μC/ml) When protein labelling was studied hemidiaphragms were preincubated 15 min with puromycin and then incubated 2 hours after addition of ^{14}C-proteinhydrolysate (0 2 μC/ml) and unlabelled glucose (1 5 mg/ml) Glycogen and protein were determined as described under "analytical procedures" Each value for glycogen is the mean of 3 experiments, the values for protein are from a single, typical experiment.

acids would become available for gluconeogenesis, with consequent dilution of the specific activity of glycogen This mechanism could hardly account for the effect of puromycin analogues, which are weak inhibitors of protein synthesis, as shown below However, to test this possibility, the effect of puromycin on the labelling of glycogen

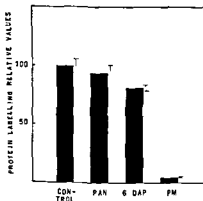


Fig 4

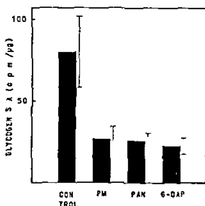


Fig 5

from ^{14}C-protein hydrolysate was examined Puromycin did not increase the ^{14}C-labelling of glycogen, as would be expected if increased gluconeogenesis was a consequence of puromycin treatment

As demonstrated in Fig 1 the effect of puromycin on the ^{14}C-labelling of glycogen was apparent throughout a two hours incubation period, during which the amount of glycogen was reduced by about 50 % (Fig 2)

Puromycin dose-response curves for the labelling of glycogen and protein are presented in Fig 3 Half maximal inhibition of the labelling of protein from ^{14}C-proteinhydrolysate was obtained at a concentration less than 20 µg/ml At a concentration of 150 µg/ml protein labelling was inhibited by 90 % increasing the concentration of puromycin to 600 µg/ml caused little further inhibition The labelling of glycogen from ^{14}C-glucose differed somewhat from this dose response relationship At a concentration of 75 µg/ml there was no effect on the specific activity of glycogen, while at higher doses an increasing inhibition was observed

A decrease in the observed incorporation of ^{14}C-glucose into glycogen might be due to depressed synthesis or increased breakdown As demonstrated in Fig 2 puromycin did not increase the breakdown of glycogen The possibility that puromycin might have a glycogenolytic effect was excluded by the following experiment Hemidiaphragms were preincubated 10 min at room temperature (20—22° C) in the presence of ^{14}C-glucose (0 5 µC/ml), and then soaked in a large volume of buffer for 30 min at 0° C After subsequent incubation for 30 min at 37 C with or without puromycin (270 µg/ml), there was no significant difference in the ^{14}C-labelling of glycogen

TABLE II. Glucose-6-P concentrations in diaphragms incubated with and without glucose and puromycin. Pa red hem [illegible]

[illegible] analytical procedures"

Addition to incubation medium	Number of experiments	Glucose 6-P
		μmoles/100 g wet tissue[1]
No addition	6	5 35±0 64
Puromycin	6	5 22±0 67
Glucose	6	18 82±1 53
Glucose + puromycin	6	21 04±2 06

[1] ± standard error of the mean

Comparative Effects of Puromycin and Puromycin Analogues

From studies on the incorporation of ^{14}C-protein hydrolysate into protein it became clear that 6-DAP and PAN had only weak inhibitory effects, while in presence of puromycin the incorporation was inhibited more than 90 % (Fig 4). In these experiments pyromucin and puromycin analogues were used in equimolar concentrations (5×10^{-4}M). By raising the concentration of PAN to 10^{-3}M a more pronounced inhibition of the ^{14}C-labelling of protein was observed.

The comparative effects of equimolar concentration of puromycin and puromycin analogues on the incorporation of ^{14}C-glucose into glycogen are shown in Fig 5. The inhibitory effects were of the same order of magnitude for puromycin, PAN, and 6-DAP.

Levels of Glucose 6-P

The effect of puromycin on the ^{14}C-labelling of glycogen was dependent on the glucose concentration of the [illegible]

glucose glycogen α 4 glucosyltransferase, EC 2 4 1 11) from muscle (Rosell Perez and Larner 1962). As shown in Table II there was a marked increase in the tissue concentration of glucose-6-P when the glucose concentration of the incubation medium was raised from zero to 2 mg/ml. Puromycin had no significant effect on the amount of glucose 6 P, neither in the absence nor in the presence of glucose.

Discussion

Since puromycin in many mammalian systems inhibits protein synthesis without apparent suppression of other metabolic processes, this antibiotic has been a useful tool to test whether hormonal effects involve protein synthesis. However, where experi-

ments with puromycin demonstrate the dependence of a hormonal effect on protein synthesis the question of the specificity of the puromycin is always important. That caution in interpretation is necessary is obvious from the finding of Hofert and Boutwell (1963) that puromycin exerts a glycogenolytic effect in mouse liver by a mechanism which appears to be different from its effect on protein synthesis. It should also be mentioned that according to Korner and Raben (1964) the insulin effect on epinephrine stimulation of fatty acid release from adipose tissue is inhibited by puromycin and even more pronounced by PAN, which causes only slight inhibition of protein synthesis. Furthermore Agosin and von Brand (1954) demonstrated an inhibitory effect of puromycin and PAN on the carbohydrate metabolism of *Trypanosoma equiperdum*, and found that this inhibition was counteracted by adenine. Eisenstein *et al* (1965) observed a depressed carbohydrate synthesis in rat liver slices after incubation with PAN. The inhibition was overcome by adding alanine to the incubation medium. These investigators however, suggested that the effect of PAN was due to an impaired synthesis of gluconeogenetic enzymes.

In the present work it has been demonstrated that the labelling of glycogen by ^{14}C-glucose is depressed by puromycin and some evidence has been obtained that this is due to an inhibition of glycogen synthesis. This effect of puromycin raises the question of the relationship to the puromycin inhibition of protein synthesis. From the comparative effects of puromycin and puromycin analogues it is obvious that 6-DAP and PAN had only weak effects on protein synthesis (Fig. 4). The effects on ^{14}C-labelling of glycogen, however, were a similar order of magnitude for 6-DAP, PAN and puromycin (Fig. 5). This would seem to indicate that puromycin interferes with glycogen metabolism by a mechanism not related to its effect on protein synthesis. The possibility is not excluded however, that the synthesis of a specific protein responsible for the control of glycogen synthesis is sensitive to 6-DAP and PAN. That PAN might be a potent inhibitor of enzyme synthesis is evident from the findings of Weber and Singhal (1964). They found that the cortisone induced rise in the activities of hepatic glucose 6-phosphatase and fructose 1,6-diphosphatase were abolished by actinomycin D and markedly inhibited by PAN.

The different dose effect relationship for puromycin inhibition of the ^{14}C-labelling of glycogen and protein should be mentioned. This observation suggests that the effects of puromycin on glycogen and protein synthesis have different reaction mechanisms. However the possibility exists that the synthesis of a specific protein responsible for the control of glycogen synthesis differs from the over all protein synthesis with respect to puromycin sensitivity.

Further evidence for the concept that puromycin inhibits glycogen synthesis by a mechanism different from its effect on protein synthesis is provided by the finding that the inhibition of the ^{14}C-labelling of glycogen could be partly reversed by adding glucose as a substrate to the incubation medium (Table I). Glucose in the incubation medium did not interfere with the puromycin inhibition of protein synthesis (Fig. 4). A raised glucose concentration might favour glycogen synthesis in different ways by providing necessary substrate, by providing energy for the formation of uridine-d-phosphoglucose and by raising the concentration of glucose-6-P, probably an important activator of glycogen synthetase *in vitro*. According to Leloir *et al* (1959) the concentration of glucose-6-P necessary for half maximal activation of glycogen synthetase from rat muscle is 6×10^{-4} M. The concentration (μmoles/mg wet tissue) found in diaphragm muscle incubated with and without glucose was 2×10^{-4} and 0.5×10^{-4}

respectively (Table II) It is likely that this change in glucose-6-P concentration is of importance in the regulation of glycogen synthesis The possibility therefore exists that although puromycin causes inhibition of glycogen synthesis when the level of glucose-6-P is low, this inhibition can not be detected at higher concentrations of this cofactor

Concerning an effect of puromycin which differs from its inhibition of protein synthesis Hofert *et al* (1962) proposed an interference with the activity of glycogen phosphorylase Puromycin might inhibit the phosphodiesterease which cleaves cyclic 3',5'AMP, by a mechanism similar to that reported for methyl xanthines such as theophylline (Sutherland and Rall 1960) This would lead to glycogen breakdown by an increased amount of phosphorylase *a* Such a mechanism could hardly account for the effect of puromycin demonstrated in the present work, where no interference with glycogen breakdown has been observed However the possibility exists that puromycin by raising the level of cyclic 3',5'AMP might inhibit the activity of glycogen synthetase Rosell Perez *et al* (1962) ... nthetase one form active in ... in the presence of glucose 6- ... 4) cyclic 3 5 AMP tends to decrease the activity of glycogen synthetase from muscle by stimulating the ATP Mg reaction leading to the glucose-6 P dependent form of the enzyme

The hypothesis that in presence of puromycin there is a conversion of glycogen synthetase from the I form to the D form might explain why the effect of puromycin on glycogen synthesis was present only at low glucose concentrations of the incubation medium At low glucose concentrations i e low tissue levels of glucose-6 P a conversion of the enzyme from I form to D form would reduce the total enzyme activity Higher glucose concentrations on the other hand would provide the necessary cofactor for the D form In this case the total enzyme activity might not be affected

Since puromycin inhibits glycogen synthesis in muscle in the absence of added hormones (possibly by a mechanism different from the effect of puromycin on protein synthesis) this antibiotic appears not to be suitable for testing whether hormonal effects on muscle glycogen depend upon protein synthesis Indirectly the observations presented here favour the view that *de novo* synthesis of enzymes is not involved in the effect of insulin on muscle glycogen (see Sovik 1965)

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References

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CARROLL, N V ... LONGLEY and J H ROE ... on of glycogen in liver and muscle by use of anthrone reagent *J biol Chem* 1956 *220* 583—593

EBOUÉ BONIS D A M CHAMBAUT P VOLFIN and H CLAUSER Action of insulin on the isolated rat diaphragm in the presence of actinomycin D and puromycin *Nature (Lond.)* 1963 *199* 1183—1184

EISENSTEIN A B M RONA and B BRUMMER In vitro effects of puromycin aminonucleoside on liver carbohydrate metabolism *Endocrinology* 1965 *76* 191—193

Hofert J and R K Boutwell, Puromycin induced glycogenolysis as an event independent from inhibited protein synthesis in mouse liver, effects of puromycin analogs *Arch Biochem* 1963 *103* 338—344

Hofert, J J Gorski G C Mueller and R K Boutwell, The depletion of liver glycogen in puromycin treated animals *Arch Biochem* 1962 *97* 134—137

Hohorst H J D-glucose-6-phosphate and D-fructose-6-phosphate Determination with glucos

Analys

Korner

ep nez

1287—1289

1962 *1* 769—772

Rosell Perez, M and J Larner Studies on UDPG-glycogen transglucosylase V *Biochemistry* 1964 *3* 81—88

Sovik O and O Walaas Insulin stimulation of glycogen synthesis in the isolated rat diaphragm in the presence and in the absence of puromycin and actinomycin D *Nature* (Lond) 1964 *202* 396—397

Sutherland E W and T W Rall, The relation of adenosine 3 ,5 phosphate and phosphorylase to the actions of catecholamines and other hormones, *Pharmacol Rev* 1960 *12* 265—299

Walaas O and E Walaas Effect of epinephrine on rat diaphragm, *J biol Chem* 1950 *187* 769—776

Weber G and R L Singhal, Role of enzymes in homeostasis *J biol Chem* 1964 *239* 521—526

Yarmolinsky M and G de la Haba, Inhibition by puromycin of amino acid incorporation into protein *Proc nat Acad Sci* (Wash) 1959 *45* 1721—1729

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On the Biological Assay of Secretin. The Reference Standard

By

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Abstract

Jorpes, J E and V Mutt *On the biological assay of secretin The reference standard* Acta physiol scand 1966 *66* 316—325 — The authors discuss the methods in use for the assay of secretin preparations in dogs cats and rats A detailed description is given of the method of Mutt and Söderberg, maintaining the cats anesthetized continuously for 4—5 days Particular attention is paid to re ent methods [illegible]

From the days in 1926 when Chiray and his coworkers Chiray *et al* 1926, 1930, Chiray and Bolgert 1936, Bolgert 1935) in France introduced secretin, as prepared by Penau and Simonnet (1925), as a diagnostic tool in medicine numerous attempts have been made to obtain stable secretin preparations to be used as reference standard in the study of the secretory capacity of the pancreatic gland In general the field seemed to be inaccessible Poorly stable preparations with only a few clinical units of secretin activity per mg and giving side reactions were mostly obtained The pharmaceutical houses, *Byla*, Paris, *Astra*, Södertalje, Sweden and *Wyeth Co*, Philadelphia, also soon dropped their manufacture of secretin while the Eli Lilly Company, Indianapolis, continued until 1961, to supply secretin on a non-commercial basis for experimental purposes

In spite of this the interest in this hormone was kept alive In continuation of the classical work performed in Sweden in the 1930 s (Hammarsten *et al* 1937, Ågren *et al* 1936, Lagerlöf 1942), a number of American authors (Diamond *et al* 1939, 1940, Lake 1947, Dornberger *et al* 1948, Friedman *et al* 1945, Dreiling 1953, 1954) found the secretin test valuable Similar experiences were reported by Bolgert (1948) in France and by Dubois (1952) in Switzerland In recent years the value of the secretin test has been amply demonstrated through the extensive studies of Dreiling and Janowitz

TABLE I Correlation between the secretin dose and the amount of $NaHCO_3$ titrated in the duodenal contents (Werner and Mutt[49]) Secretin test in 7 normal persons, weighing 62—70 kg

	Secretin µg/kg	Ml 0 1 N alkali	
1	5	81	(in ³/₄ hr)
	10	127	(in ³/₄ hr)
2	5	52	(in ³/₄ hr)
	15	165	(in ³/₄ hr)
3	10	146	(in 1 hr)
	20	329	(in 1 hr)
4	7 5	73	(in 1 hr)
	15	158	(in 1 hr)
5	7 5	114	(in 1 hr)
	15	229	(in 1 hr)
6	5	42	(in 1 hr)
	15	120	(in 1 hr)
7	10	134	(in 1 hr)
	2 5	67	(in 1 hr)

(1957) Dreiling *et al* (1960) in New York, Raskin *et al* (1958) Wenger and Raskin (1958) in Chicago, and Sun and Shay (1957, 1960) Shay *et al* (1961) in Philadelphia, particularly since it has been shown that cancer of the pancreas (Dreiling *et al* 1960, Raskin *et al* 1958, Wenger and Raskin 1958), the gallbladder and the bile ducts (Dreiling *et al* 1960) and to a certain extent also some processes involving the liver (Henning *et al* 1960) can be diagnosed by means of exfoliative cytology on the duodenal contents after secretin injection preferably combined with a cholecystokinin-pancreozymin test. The recent development in this field has been reviewed by the present authors (Jorpes and Mutt 1959, 1960, 1961, 1962, 1964) The need for secretin is, consequently, evident and thereby also the need for reliable methods of assay and for a reference standard

Methods suggested for the biological assay of secretin

Ivy and Janecek (1959) recently described the details of Ivy's methods for assaying secretin and cholecystokinin in dogs and cats The dog unit for secretin was defined by Ivy as that amount of dried material in normal saline solution which, when injected i v in an anesthetized dog weighing from 10 to 20 kg during 10 or 15 sec, will cause a 10 drop (0 4 ml) increase in the rate of flow of pancreatic juice from the cannulated duct during a period of 10 min, the control or basal flow not being more than one drop in 2 min The secretin product is required to be free from vasodilatin The Ivy cat unit for secretin was defined in similar terms one dog unit corresponding to 2 cat units

Instead of measuring the volume of pancreatic juice secreted Hammarsten *et al* (1928) introduced the principle of titrating the alkali the amount of alkali secreted

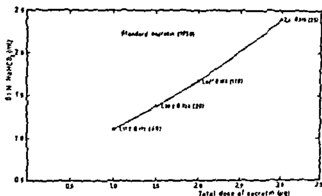


Fig 1 Relationship between secretin dose injected in the anesthetized cat and the amount of alkali secreted in 15—20 min

with the pancreatic juice being within the straight line part of the dose-response curve as well in cats (Wilander & Ågren, 1932) as in man (Hammarsten *et al* 1937) almost stoichiometrically proportional to the dose of secretin injected Werner and Mutt (1954) in titrating the bicarbonates in human duodenal contents after administering secretin found in 5 out of 7 cases with almost ridiculous accuracy twice and three times as much bicarbonate after a corresponding increase in the secretin dose injected (Table I)

The assay of our secretin standard containing 8,400 HCU per mg in a large number of cats showed almost the same close proportionality (Fig 1)

The titration of the alkali excreted with the pancreatic juice consequently gives an adequate figure for the secretin activity

The Hammarsten cat unit (HCU) of secretin is defined as the amount of secretin, which in the cat induces secretion of 0 1 ml of 0 1 N bicarbonate in the 15 min period following injection

The cat method

The technique of performing the assay in cats was very much improved by Mutt and Soderberg in 1959, who kept the cats in good condition under Placidyl anesthesia for up to 4—5 days Thereby the necessity of time-consuming daily operations was eliminated Moreover, the animals are affected for some hours after operation and react sluggishly to secretin Consequently, if they are sacrificed in the evening of the day of operation, they are discarded just when they have become useful Since, furthermore, the anesthesia produced with Placidyl (Abbott) lasts for at least 12 hrs the animals can with adequate temperature control safely be left without supervision overnight, thus making legislative regulations on this point demanding supervision superfluous

This technique can permit some 50 tests to be performed in the same animal At any time of the day an approximate value for the secretin activity as compared with a standard can be obtained within the course of 1 hr The cat functions with an astonishing precision like an ordinary titration machine Since the method has been published [illegible] will be reproduced here

[illegible] hrs before operation, but are

[illegible] th 0 6 ml per kg of Nembutal

Abbott, veterinary solution, intrapleurally Before operation, all instru[illegible]s as well as the hands of the operator, are disinfected with a 0 1 % aqueous soluti[illegible] cetylpyridinium chloride This is rinsed off with sterile water The saphenous vei[illegible]nnulated with soft plastic tubing, outer diameter 1 4 mm (Portex no 1 E),[1] exce[illegible] the site of ligation where a thin glass tube is interposed

Most of the hair on the abdomen is shorn off, and the remainder re[illegible]ve[illegible] with a depilatory lotion The abdomen is opened by a midline incision A flap of th[illegible]mach wall is fastened into the upper part of the incision Through an incision in [illegible]is flap a soft rubber tube, outer diameter 3 mm, is introduced into the stomach and a soft plastic tubing, of the same type as that used for cannulation of the vein, into the jejunum, The stomach tube is used to evacuate the gastric juice, and the jejunal tube to introduce solutions into the intestine The common bile duct could easily be cannulated and the bile led past the duodenum directly into the jejunum, by connecting the cannula to the jejunal tube This seems, however, to be superfluous, since — contrary to what might be expected from the reports in the literature — no secretion of pancreatic juice takes place in starved animals with an uninterrupted biliary flow, unless secretin is injected

A thread is passed between the pancreas and the duodenum, about 2 cm on either side of the entry of the bile duct into the duodenum With the help of these loops of thread, the part of the intestine into which the pancreatic ducts empty is pulled out and attached to the left side of the abdominal wall The main pancreatic duct is identified either in its passage from the gland to the intestine, or in the duodenal wall It is freed from extraneous tissue and cannulated — as far as possible from the gland, sometimes even in the duodenal wall — with about 2 5 mm of a 15 mm long glass tube, outer diameter 1 mm The duct is tied around the cannula The free end of the tube is connected to about 30 cm of soft plastic tubing of the same type as that used for cannulating the vein and for the jejunal tube The plastic tubing is threaded loosely once around the intestine and then passed through a stab wound in the lateral abdominal wall, leaving about 10 cm of tubing outside and a coil of 20 cm inside This prevents the cannula from being dislodged by respiratory movements The intestine is returned to the abdominal cavity, and is kept close to the incision in the abdominal wall by attaching the two threads surrounding it to the wall The incision is then closed

When the effect of the Nembutal wears off, 0 1 ml of Placidyl Abbott per kg b w is introduced into the jejunum This dose usually keeps the animals asleep for at least 12 hrs About once every 12 hrs the bladder is emptied by slight manual pressure over the appropriate part of the abdomen Tests are usually run between 8 a m and 10 p m Before each night period the animals are given 0 15 ml of a 5 % heparin solution i v , 250,000 I U of benzylpenicillin sodium i m , 20 ml of 5 % glucose slowly intrajejunally, as well as 0 1 ml of Placidyl per kg of body weight intrajejunally The same treatment except for the Placidyl is repeated in the morning Nembutal can be given instead of Placidyl but the animals cannot then be left overnight without supervision The temperature of the animals measured rectally is kept as close as possible to 37 °C by appropriately placed lamps

The assay All solutions of secretin are prepared in sterile physiologic saline, to which hydrochloric acid has been added to a concentration of 0 001 N The solutions are distributed into meticulously cleaned sterile glass test tubes and quickly frozen The contents of a tube are thawed immediately before use That secretin in solution is

[1] Manufactured by Portland and Plastic Ltd Hythe Kent England

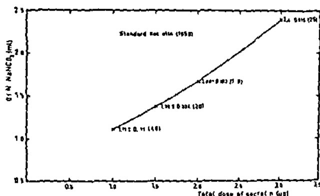


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Preparation of the animals The cats are starved for about 36 hrs before operation, but are allowed water *ad libitum* They are initially anesthetized with 0 6 ml per kg of Nembutal

the variability in the animal's reaction during the course of 2 hrs Two consecutive injections are liable to give more reliable responses allowing a 10 per cent differentiation in strength with fewer series Furthermore the 1 2 ratio of the doses is not selected for physiological reasons but in order to facilitate the mathematical treatment of the material which evidently should be of secondary importance

In all 3 cats the same results were obtained namely, 0 1 ml of the test solution equals 4 µg of the Standard or 33 6 HCU One ampoule of Secretin Vitrum No 222010 (1962) thus contains 1 680 HCU or *84 clinical units of secretin*

The rat test A technique for using rats was elaborated by Love (1957) of the Oxford group In rats starved overnight and anesthetized with urethane, pancreatic juice was collected from a cannula in the terminal part of the bile duct and measured in a calibrated capillary In some instances the bile was by passed through another cannula from the upper part of the choledochus implanted into the first portion of duodenum The basal secretion was measured before the injection and after cessation of the secretin stimulation The total volume of secretion under secretin stimulation minus the basal secretion was taken as the response Over a certain range 1 8—5 5 HCU per animal 1 v the dose/response curve was linear with variations in the slope of the curve for different animals At least 2 standard doses of secretin differing in strength by 1 3 were used for plotting the dose/response curve on which the doses of the unknown were read off Another and better alternative is to choose the dose of the unknown as close as possible to that of the standard

The technique of the rat test has recently been reinvestigated by Heatley (1965) There is a responsiveness within a 64 fold range (0 9—57 HCU), the log dose response curve being roughly linear A linear dose/response curve was obtained over a 3 fold range 1 8—5 5 HCU in accordance with Svatos and Jelinek (1957) who found the relation to be linear over a 4 fold range As found by other authors they also found frequent changes in the sensitivity of the rats causing fluctuations in the response to secretin The operation technique was simple taking for one animal less than half an hour 4—5 animals being used simultaneously Operation mortality 4—5 per cent with no difference between male and female rats 3—6 hrs after preparation the basal secretion was in a series of 68 urethanized rats 2 60 ± 0 84 µl/min/kg and the mean pancreatic response (total volume minus basal secretion) 20 0 + 5 39 µl after 5 5 HCU and 11 1 ± 3 4 µl after 1 8 HCU Mean duration of the response about half an hour

In comparing the strength of the rat unit with that of the Hammarsten cat unit counting with 8 000 HCU/mg of our reference standard one rat unit was found to correspond to 4 48 HCU Exactly the same figure 4 4 HCU = 1 rat unit had been found by Newton *et al* (1959) in using one of our previous standards having 730 HCU per mg

The most extensive study of the secretin assay in rats has recently been made by Vaille *et al* (1962) and Debray *et al* (1962 a b) They counted the number of drops of pancreatic juice secreted under the influence of secretin during the course of 1 hr and subtracted the basal secretions There was a linear relationship between the response and the logarithms of the dose applied The basal secretion from the pancreas which by Grossman (1958) was found to be as high as 0 6 ml per hr and kg was in these series probably due to the operation technique with no ligation of the pylorus several times higher than in the series of Love (1957) Heatley (1965), Svatos and Jelinek (1957) and Svatos *et al* (1960 The accuracy of the analysis was thereby reduced In order to get satisfactory accuracy Vaille *et al* 1962 p 233) found the administration of two doses

of the test and the standard in the ratio 2 3 each of them to four rats not sufficient, twelve animals being necessary

Seeing that the strength of the Ivy dog unit of secretin equals that of the clinical unit, suggested by Ågren and Lagerlöf, Debray *et al* (1962 b p 574) were anxious to choose an equal strength of the rat unit They found 6 clinical units of secretin to be necessary in order to produce under definite experimental conditions in a 250—300 g male Wistar rat, anesthetized with urethane, a secretion of 9 25 drops of pancreatic juice per hour, giving a twice as large secretion during the hour following the injection of secretin as that during the hour before injection The rat unit was therefore defined by them as 1/6 of this dose, making one rat unit in their terminology equal to the clinical unit The rat unit of the Oxford group was 1/4—1/5 of the clinical unit

Evidently any of the methods discussed above can be used for the assay of secretin The dog method of Ivy which measures the volume of the juice secreted gives accurate values, but is suited only for physiological laboratories with ample housing facilities and trained personnel The rat method as elaborated by Love and Heatley, by Debray *et al* and by Svatos and Jelinek can be a satisfactory substitute when cats are not accessible The number of animals suggested by Vaille *et al* (1962) would, however, make the assay time consuming

The cat method as elaborated by Mutt and Söderberg seems to us to be an ideal method in every respect It makes an approximate estimation of the strength of a sample possible in 1 hour and an accurate assay in a few hours There is no basal secretion and the relationship between the dose of secretin injected and the amount of alkali secreted is nearly stoichiometrical

The interrelationship between the different units suggested for the expression of secretin activity

Lagerlof (1942) used 16 cat units (HCU) per kg b w as the standard dose in the secretin test in man thereby defining the clinical unit of which one unit is ordinarily given per kg b w In analyzing the Swedish secretin Pancreotest, manufactured by Astra, Sodertalje, and stated by the manufacturer to contain two clinical units per mg, Greengard and Stein (1941) found that one dog threshold dose of Ivy corresponded to one clinical unit of Lagerlof The relative magnitude of the Ivy unit and the Hammarsten unit (HCU) was determined by Greengard and Ivy (1938) They found one Ivy dog threshold dose to be equivalent to about 20 HCU, which means that 1 clinical unit equals 20 cat units For the sake of convenience we prefer to use this relationship 1 20 instead of 1 16 as suggested by Lagerlöf

Crick, Harper and Raper (1949) used the strength of 0 1 mg of an arbitrarily chosen standard preparation of their own as a secretin unit The average amount of pancreatic juice found by them to be secreted in the 12 min period following the i v injection of 0 1 mg of this preparation in cats, anesthetized with chloralose was 1 2 ml The strength of the Crick, Harper and Raper unit will thus be about 12 Hammarsten cat units or about half of the clinical unit

The rat unit suggested by Love of the Oxford group is according to Newton *et al* and Heatley equal to 4 5 (4 48) HCU or about a quarter of the clinical unit The rat unit by Vaille, Debray *et al* has the same numerical strength as the clinical unit and the Ivy dog threshold dose

The clinical unit of secretin is thus fairly well defined in relation to the other units suggested It was introduced by Lagerlof in Sweden in the 1930 s as being of a suitable size for the administration of 1 unit per kg b w in the routine secretin test for pancreatic function in man and was independently applied by American authors as the Ivy dog

threshold dose It has, furthermore, recently been accepted by the French group working under Debray There can consequently be no discussion on an international level about using other units than the clinical unit of Lagerlof for the expression of the secretin activity

The secretin standard

In the past different authors used their own reference standards varying in strength from that of the acetone-dried duodeno jejunal mucosa of the dog (Burn and Holton 1948) to preparations with a few, possibly up to 10—20 clinical units per mg In 1953 a conference on the assay of secretin was held in Montreal attended by representatives from 8 countries (Friedman 1954) No standard nor any international unit was established The secretin, available at that time, was too labile for the purpose

Because of the lack of a stable standard preparation of secretin from the past there will be no possibility of comparing the strength of the presently available material with that of the earlier preparations However, since the numerical value of the clinical unit can be determined both with the cat, dog and rat methods, there will be no greater difficulty in defining the strength of the new standard

The greatest obstacle against creating a secretin standard is the lability of the material Air dried preparations of the crude secretin lose activity when stored at room temperature The same applies according to our experience also to purified lyophilized preparations

Our first two standard preparations precipitated with acetone and ether suffered considerable loss in activity during one year In August 1958 we lyophilized the hydrochloride of a fairly active preparation with 420 clinical units (8400 HCU) per mg and distributed it by weighing 1 mg portions into sterile test tubes with ground stopcocks Part of the material was stored at room temperature, the remainder at —20° C In order to prevent loss of activity in treating the solutions the samples were handled in a definite manner The content of a test tube was dissolved in 10 ml sterile 0 01 N HCl and distributed on 10 sterile test tubes 1 ml in each After filling up to 10 ml with sterile physiological saline — thus 10 μg per ml, the solution was deep frozen and stored at −15° C. Before use it was thawed and distributed on 5 ml sterile test tubes to be kept in the ice box during the course of an experiment

The activity was determined in cats as described by Mutt and Söderberg (1959) In 209 single tests on cats the following results were obtained (Fig 1) 1 μg of the standard produced 1 11 $\pm$ 0 17 ml of pancreatic juice in the anesthetized cat (46 injections), 1 5 μg produced 1 38 $\pm$ 0 32 ml (20 injections), 2 μg produced 1 68 $\pm$ 0 163 ml (118 injections) and 3 μg produced 2 40 $\pm$ 0 51 ml (25 injections)

The figure 1 68 ml obtained with 2 μg corresponds to 420 clinical units (8 400 HCU) per mg In some connections the strength 8 000 HCU per mg has been given for this standard Preferably the figure 8 400 should be used

Our first impression was that this standard had preserved its activity fairly well as dry powder at room temperature in closed test tubes After 4 years, however, it was evident that the sample kept at room temperature gave a far too weak response in the cats, the sample kept as dry powder at — 20 C being twice as active

We also made an attempt to use as reference standard pure porcine secretin polypeptide, lot No 222010 (1962) of the commercial secretin, Secretin Vitrum, manufactured by AB Vitrum, Franzéngatan 9 Stockholm Sweden being selected for the purpose It was stored at room temperature However it also proved not be stable. In 3 years the activity had dropped from 84 to 15 clinical units per ampoule

As the facts stand therefore it will be necessary to check the activity of the reference standard with regular intervals using any of the biological methods So far, we know that the acetate form is very unstable, particularly in solution It remains to study the stability of the secretin hydrochloride under anhydrous conditions at low temperature in an oxygen free atmosphere

References

ÅGREN, G , LAGERLÖF, H and BERGLUND, H , The secretin test of pancreatic function in the diagnosis of pancreatic disease *Acta med scand* 1936 *90* 224—271

BOLGERT, M , *Lésions du pancréas et troubles fonctionnels pancréatiques* Diagnostic en clinique par l'épreuve a la sécrétine purifiée Thèse de Paris 1935 (Masson)

BOLGERT, M , Dix ans de practique de l'épreuve à la secrétine dans les affections du pancréas. *Acta chir belg* 1948 *47* Suppl 2 453—458

BURN, J H and HOLTON P The standardization of secretin and pancreozymin *J Physiol* (Lond) 1948 *107* 449—455

CHIRAY M and BOLGERT, M Le diagnostic des affections pancreatiques par l epreuve a la sécrétine purifiée *Presse méd* 1936 *44* 428—431

CRICK, J HARPER A A and RAPER, H S , On the preparation of secretin and pancreozymin. *J Physiol* (Lond) 1949 *110* 367—376

DEBRAY, CH VAILLE CH DE LA TOUR, J ROZÉ CL and SOUCHARD M , Action des sécrétines du commerce sur la sécrétion pancréatique externe du rat *J Physiol* (Paris) 1962 *54* 519—577

DEBRAY CH DE LA TOUR J , VAILLE, CH , ROZÉ CL and SOUCHARD M Contribution à l étude de la sécrétion biliaire et pancréatique externe chez le rat *J Physiol* (Paris) 1962 *54* 459—499

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ses with

118

182—187

H Pancreatic

671—675

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logy

test in the diagnosis of pancreatic and biliary tract cancer *Med Clin N Amer* *44* 801—815

DUBOIS, J -P , L'insuffisance pancréatique externe étudiee par l epreuve à la sécrétine Thèse Fac Méd Univ Genève (Karger, Bâle 1952) *Gastroenterologia* 1952 *78* 169—192

FRIEDMAN, M H F and SNAPE, J , Comparative effectiveness of extracts of intestinal mucosa in stimulating the external secretions of the pancreas and the liver *Fed Proc* 1945 *4* 21—22

Friedman M H F, The significance of the discovery of secretin *Gastroenterology* 1954 *26* 795—801
Greengard H and Stein I F Assay of secretin *Proc Soc Exp Biol* (N Y) 1941 *46* 149—151
Greengard H and Ivy A C, The isolation of secretin *Amer J Physiol* 1938 *124* 427—434
Gr
Ha
Ha
He

scand 1959 *45* 220—230
Jorpes, J E and Mutt V Secretin, Pancreozymin and Cholecystokinin *Gastroenterology* 1959 *36* 377—383
Jo
Jo

Jorpes J E and Mutt V, On the biological activity and amino acid composition of secretin *Acta chem scand* 1961 *15* 1790—1791
Jorpes E und Mutt V, Die Anwendung von Sekretin und Cholecystokinin Pankreozymin *Klin Wschr* 1962 *40* 661—665
Jorpes J E and Mutt, V *Gastrointestinal hormones* Pincus Thimann Astwood The Hormones IV *Acad Press* 1964 365—383
La
La
Le
184
M *J Physiol*
68
of secretin
Penau H and Simmonet, H Sécrétine duodénale et insuline *Bull Soc* (Paris) *Chim biol* 1925 *7* 17—25
Raskin H F The diagnosis of cancer
secretory methods
tic function profile
142
duodenal contents
after secretin in the diagnosis of diseases of the pancreas *Gastroenterology* 1957 *32* 212—231
Sun D C H and Shay H Pancreozymin secretin test The combined study of serum enzymes and duodenal contents in the diagnosis of pancreatic disease *Gastroenterology* 1960 *38* 570—581
Svatos A and Jelinek V Gastrointestinal hormones Titration of secretin in rats *Physiol Bohemoslov* 1957 *6* 220—223
Svatos V Kozlik V Verisova Z Titration of secretin in small laboratory animals *Physiol Bohemoslov* 1960 *9* 90
Wenger J and Raskin H F The diagnosis of cancer of the pancreas biliary tract and

Acta physiol. scand. 1966. 66. 326—332

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The Effect of Cinchophen on the Acid Polysaccharides of the Gastric and Duodenal Wall in Dog

By

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Received 20 August 1965

Abstract

[illegible] were fractionated and the aminosugar content of the fractions was determined. After [illegible] days cinchophen feeding a significant increase in the amount of fractions containing chondroitin sulfuric acid B and in three fractions containing epithelial sulfopolysaccharides were noted whereas the amount of the fraction containing hyaluronic acid showed an opposite tendency. The change in chondroitin sulfuric acid B content was the greatest in the middle sections of the mucosa of the antrum. At this time no ulcers had yet appeared.

After 14 days feeding the content of chondroitin sulfuric acid B fell significantly in all parts [illegible] noted [illegible] er the [illegible] hanges [illegible] inophen [illegible] d and [illegible] ulcer [illegible] formation.

Cinchophen, an agent known to produce experimental gastric ulcers in dogs (Bollman, Stalker and Mann 1938), was observed to stop almost completely the secretion of duodenal mucus in duodenal pouch dogs (Hartiala, Ivy and Grossman 1950). Since this observation was made, work in our laboratory has been concentrated on the study of gastric and duodenal mucosubstances. The lack of suitable methods prevented, for a long time, a study of these mucosubstances. However, the incorporation of radioactive sulfate ($^{35}SO_4$) into the gastrointestinal mucosa and mucus as an ester sulfate was noted in the 1950s (Boström and Odelblad 1954, Dziewiatkowski 1956, Jennings and Florey 1956 and Kent, Whitehouse, Jennings and Florey 1956), this was thought to be mainly as the sulfomucopolysaccharide. Using this knowledge, Häkkinen (1961) studied the [illegible] the sulfate content and ^{35}S uptake in the antrum and duodenum in guinea [illegible] e insufficient for ulcer production. This [illegible] ontent of the tissue and the ^{35}S content

changed in the same way Repeated injections of histamine (4 to 9 injections) regularly produced duodenal ulcers and a significant diminution in sulfate content

The results of this study indicate that biochemical changes occur in the gastrointestinal wall beyond the location of the ulcer itself It has not been established to what extent the changes in the sulfate content reflects changes in the connective tissue polysaccharides, on the one hand, and in the macromolecules of the secretion, on the other The idea that chondroitin sulfate, hitherto known only as a connective tissue polysaccharide could be secreted as a mucous component in the gastric and duodenal juices is difficult to conceive off According to the present concepts chondroitin sulfate is synthetized by the fibroblasts (Schubert 1964) On the other hand, radioautographic studies have shown that the radioactive sulfate is incorporated not only in the connective tissue but also in the mucosal neck cells and the cells of Brunner's glands and that it is secreted from here into the lumen (Bostrom and Odelblad 1954, Dziewiatkowski 1956 Jennings and Florey 1956 and Kent *et al* 1956) It may thus be expected that the sulfopolysaccharide of the mucus would differ from that of the connective tissue namely chondroitin sulfate

The difficulties encountered in the isolation and characterization of the mucosal polysaccharides derive from the fact that the samples have contained too many different types of polysaccharides to allow the isolation of individual components Scott 1960 has described a new method for precipitation and fractionation of the acid polysaccharides This has been intended to be employed mainly for the analysis of the connective tissue polysaccharides such as hyaluronic acid, chondroitin sulfate and heparin We have applied this method also for the identification of the polysaccharides of the gastric wall and mucus This information has been published elsewhere (Hakkinen et al 1965) It was necessary to distinguish the individual mucosal polysaccharides from one another and to characterize them Such fractionation is a prerequisite before any quantitative colorimetric analyses such as aminosugar determinations can be succesfully used to show possible changes in the mucosal polysaccharides Recent literature seems still to offer investigations in which direct aminosugar determinations have been employed for the unfractionated gastric secretions or wall constituents In our opinion such results cannot reflect actual biological changes in the various polysaccharide compartments as has been claimed (Robert Bayer and Nezamis 1963 and Robert and Nezamis 1963)

Three different types of polysaccharides were isolated namely hyaluronic acid chondroitin sulfate B and a previously unknown sulfate-containing polysaccharide The last named is not entirely unknown however as far as the elementary constituents are concerned It resembles that general type which is found in the blood group polysaccharides and most epithelial polysaccharides except that these lack the sulfate radical The incorporation of sulfate into these has not been known to occur The molecular analyses revealed glucosamine galactosamine, galactose fucose and sialic acid Four different fractions of this polysaccharide were obtained which differed among each other only in the concentration of sulfate The residual peptide fragments still present after the papain hydrolysis have not been studied in detail As judged from the nitrogen analyses, their molar amounts are not great

The sulfate-containing epithelial polysaccharide under consideration proved not to contain uronic acid For the purpose of comparison we also isolated the acid polysaccharides from human gastric juice (Hakkinen *et al* 1965) These were found to be identical with those isolated from the gastric wall of the dog In the gastric juice not even

traces of chondroitin sulfate or hyaluronic acid could be detected It would, therefore, be likely that this new sulfopolysaccharide is derived from the secreting epithelial cells of the gastric wall. Structually it is entirely different from the sulfopolysaccharide of the connective tissue, chondroitin sulfate. In the present work we are dealing with gastric and duodenal tissue Consequently the main interest is focussed particularly on the connective tissue polysaccharides of the wall and on possible changes in these The secreted polysaccharides of the mucus should be determined directly from the secretions Determination of these constituents from the tissue contents does not give us information about the changes in the actual secretion or synthesis The analyses of these epithelial polysaccharides have nevertheless been included in our data since previous information of their contents in the tissue at certain moments are not available.

Materials and methods

Young female dogs weighing from 9 to 10 kg were used The animals were fed ad libitum — the [illegible]

stomach together with a 5 cm strip of duodenum was excised and promptly refrigerated the stomach contents and omental fat were discarded and any erosions and ulcers were noted

The stomachs of the first group were divided into two parts by cutting along the border between the antrum and corpus The duodenum was used as the third sample The wall was homogenized — all layer[illegible]
digestion The homogenat[illegible]
its own enzyme activity [illegible]

In the second group the study was concerned with the antrum the area where the cinchop[illegible] ulcers are almost all localized The mucosa in this part was not difficult to dissect away from the muscular layer This piece of mucosa was flattened and promptly deep frozen on solid CO_2 [illegible] were taken using a hammer and [illegible] roup was weighed homo- [illegible] The samples in the second [illegible]

— [illegible] series

It is possible to precipitate the acid polysaccharides or polyanions quantitatively with cetylpyridinium chloride (CPC) from an aqueous solution representing an extract of the gastric tissue In the supernatant remain the neutral polysaccharides

The neutral polysaccharides remain in the supernatant provided that the solution contains such salts as $MgCl_2$ or NaCl over the so-called critical concentration at pH about 6 This is characteristic for each cetylpyridine complex of the polyanion

After they were liberated from their CPC complex by reprecipitation with alcohol from $MgCl_2$ solutions the acid polysaccharides were fractionated in small columns containing [illegible] 100 μg in 6 × 0.25 [illegible] nated with 1 % CPC [illegible] bed elsewhere [illegible] he salt concentrations [illegible] aCl elution carries a

TABLE I The effect of cinchophen on the acid polysaccharides of the gastric wall in dog, after feeding for 14 days The results are expressed as μg aminosugar g tissue wet wt The fractions are characterised by the normality of salt solution used for the elution (for details see text)

Fraction	Control dogs	Cinchophen dogs	Significance of difference
a) *Antrum*			
0 3	17 3 ± 7 4	12 9 ± 6 0	0 35 > P > 0 30
0 5	17 2 ± 5 3	10 7 ± 1 4	0 15 > P > 0 10
0 6	9 0 ± 1 7	7 8 ± 1 4	0 40 > P > 0 35
0 8	20 2 ± 2 2	12 3 ± 1 7	0 01 > P > 0 005
1 0	35 7 ± 8 5	15 6 ± 1 7	0 025 > P > 0 0125
1 2	11 8 ± 1 7	5 6 ± 1 0	0 01 > P > 0 005
b) *Duodenum*			
0 3	42 6 ± 21 3	10 1 ± 2 2	0 1 > P > 0 05
0 5	17 6 ± 4 9	12 4 ± 2 8	0 20 > P > 0 15
0 6	10 1 ± 2 6	6 9 ± 2 4	0 20 > P > 0 15
0 8	14 2 ± 2 4	5 6 ± 2 2	0 025 > P > 0 0125
1 0	31 1 ± 7 4	10 4 ± 3 9	0 025 > P > 0 0125
1 2	9 6 ± 2 8	9 1 ± 1 7	0 45 > P > 0 40
c) *Corpus*			
0 3	51 1 ± 9 9	30 7 ± 9 5	0 1 > P 0 05
0 5	32 2 ± 9 9	26 1 ± 4 6	0 30 > P > 0 25
0 6	16 0 ± 4 6	12 0 ± 3 5	0 25 > P > 0 20
0 8	17 4 ± 2 8	14 2 ± 3 6	0 30 P > 0 25
1 0	35 0 ± 7 7	13 3 ± 3 3	0 025 > P > 0 0125
1.2	10 2 ± 4 5	4 8 ± 1 4	0 20 > P > 0 15

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carry fractions of epithelial sulfopolysaccharide each depending on the sulfate content of the component 1 0 and 1 2 N MgCl, eluates contain a substance identical with chondroitin sulfate B The fractionation into these two components was considered to be due to their different sulfate contents The salt normalities used in the elution procedure are used as the names of the fractions

No carbohydrate containing material could be found in any fractions using stronger or weaker salt solutions than these The recovery from the column us ng this method was almost 100 %, however it must be stressed that great care must be taken when us ng this technique — especially with the pack ng of the column

The fractions were precipitated by diminish ng the salt concentration to a point below the critical CPC precipitati n point The precipitate was hydrolysed in 6 N HCl at 100 ° C f r 8 hrs and the aminosugar c ntent was determ ned after evaporation of HCl using the

TABLE II The effect of 6 days feeding with cinchophen on the acid polysaccharide content of antral mucosa of dog expressed as in Table I

Antrum Fraction	*Control dogs*	Cinchophen dogs	Significance of difference
0.3	*76.0 ± 7.2*	*55.8 ± 12.3*	0.1 > P > 0.05
0.5	*22.9 ± 2.6*	*31.5 ± 6.7*	0.025 > P > 0.0125
0.6	*13.7 ± 1.0*	*25.1 ± 13.1*	0.01 > P > 0.005
0.8	*20.4 ± 1.4*	*35.1 ± 2.8*	P < 0.0005
1.0	51.7 ± 6.9	72.5 ± 2.8	0.0125 > P > 0.01
1.2	*24.6 ± 3.0*	38.9 ± 5.1	0.0025 > P > 0.0005

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Results

Series I (cinchophen for 14 days) The antrum of every dog showed ulcers reaching to the deep muscularis. No dog had visible lesions in the corpus or duodenum. The fractions obtained with the cinchophen dogs showed a decreased amount of aminosugar compared to the control dogs (Table I). The greatest and the most significant change was found in the antrum in the 0.8 N, 1.0 N and 1.2 N fractions. The 0.3 N, 0.6 N and 0.8 N fractions in the corpus did not differ significantly from the controls.

The diminution in the polysaccharide content produced by the cinchophen is largest in the 1.0 N fraction, over 50 %, and this change is the same in the corpus, antrum and duodenum.

Series II (cinchophen for 6 days) Every dog showed erosions in the antrum. These were probably developing ulcers. The samples were from the visible healthy tissue, not from the ulcer.

The results of the quantitative analyses of the acid polysaccharides are shown in Table II expressed again as aminosugar. These results are from the mucosa separated from the muscularis and serosa, so the amounts of the polysaccharides are greater than in the first series.

The results for this series differ from those of the first series in that the 0.5 N, 0.6 N, 0.8 N, 1.0 N and 1.2 N fractions in the cinchophen dogs were about 30 % greater than in the control dogs.

The fractionation of the layers of tissue showed the cinchophen effect only in the two largest fractions, 0.3 N and 1.0 N. The 0.5 N, 0.8 N and 1.2 N fractions are found in the whole mucosa equally distributed. The 0.3 N and 1.0 N fractions in the middle of the mucosa (sections 4, 5 and 6) were relatively higher after cinchophen feeding.

Discussion

The connective tissue polysaccharides of the gastric wall, chondroitin sulfate B and hyaluronic acid according to our analyses, are not released from the wall. They more likely form a structural compartment of its supporting tissue. How these substances

are precisely arranged in the gastrointestinal wall has not been clai ied In other parts of the organism they are known to form a gel, an interstitial su stance in the collagenous network Perhaps they also connect the epithelial cells t th s network All transport between the cells and capillaries finally goes through this gel From this point of view the observed changes in the connective tissue polysaccharides in the gastroduodenal wall are reasonable It might be added, in this connection that the role of HCl and pepsin in the production of ulcer under these conditions must also be considered As has been shown in totally gastrectomized dogs cinchophe does not alone without the presence of HCl, result in the final ulcer formation Hartiala and Antila 1963)

In this study we look at two stages in the developing ulcer early ulceration and the first stages of chronic ulcer The results show that the polysaccharide content in the early stage is increasing This is true for both the epithelial mucuspolysaccharides and the connective tissue polysaccharides The tissue samples are from the healthy mucosa so from these results we can hope to find the same for the whole antrum

In the chronic experiment we found that cinchophen caused a decrease in the polysaccharide content especially in the connective tissue polysaccharides

The most surprising observation is that the content of chondroitin sulfuric acid decreased evenly in the whole stomach from the duodenum up to the upper corpus It may be supposed that in the early stage there is a reactive stimulation and that further feeding of cinchophen produces exhaustion The result is analogous to the earlier experiment in guinea pigs where the sulfate content in the distal stomach and duodenum was changed in the same manner after histamine injection The results in an experiment with restraint rats showed the same tendency (Hakkinen Hartiala and Lang 1965)

It is obviously too early to speculate on what actually happens in the gastroduodenal ulcer area during these two different conditions The histological and even macroscopic appearance of these experimental ulcers is edematous Water accumates in the extra cellular connective tissue The edema is not confined only to the ulcer location but extends to larger areas Whether the polysaccharide changes and edema have something in common remains to be proven That the connective tissue polysaccharide gel incorporates water and electrolytes is known (Katchalsky 1964)

This study has been supported by a Public Health Service grant No AM 6018

References

BOLLMAN J L L K STALKER and F C MANN Experimental peptic ulcer produced by cinchophen *Arch intern Med* 1938 *61* 119—128

BOSTROM H and E ODELBLAD An autoradiographic study on the occurrence of injected rad sulphate in the intestine *Acta physiol scand* 1954 *32* 124—128

DZIEWIATKOWSKI D D Turnover of S^{35} sulfate in the mucosa of the gastrointestinal tract of rats as seen [illegible]

GARDELL, [illegible] chemical Analysis Intersc [illegible]

HARTIALA [illegible] nism of cinchophen ulcer [illegible]

HARTIALA, K A C IVY and M I GROSSMAN Effect of feeding cinchophen on secretion of juice by duodenal glands in dog *Amer J Physiol* 1950 *162* 110—114

HAKKINEN I Histamine induced changes in the sulphate metabolism of the duodenum and prepyloric region of the stomach an experimental study with special reference to ulcer formation *Acta physiol scand* 1961 *51* Suppl 177

HAKKINEN, I., K. HARTIALA and H. LANG, The effect of restraint on the content of acid polysaccharides of glandular gastric wall in rat. *Acta physiol. scand.* 1966. *66.* 333—336.

HAKKINEN, I., K. HARTIALA and T. TERHO, The fractionation and characterisation of the acid polysaccharides of the gastric wall in dog. *Acta chem. scand.* 1965. *19.* 800—806.

HÄKKINEN, I., K. HARTIALA and T. TERHO, The fractionation and characterisation of acid

KENT, P. W., M. W. WHITEHOUSE, M. A. JENNINGS and H. W. FLOREY, Observations on the incorporation of S^{35} in duodenal mucosubstances. *Quart. J. exp. Physiol.* 1956. *41.* 230—246.

Acta physiol. scand. 1966. 66 333—336

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The Effect of Restraint on the Content of Acid Polysaccharides of Glandular Gastric Wall in Rat

By

I Häkkinen, K Hartiala and H Lang

Received 20 August 1965

Abstract

Hakkinen I, K Hartiala and H Lang *The effect of restraint on the content of acid polysaccharides of glandular gastric wall in rat* Acta physiol scand 1966 66 333—336 — Rats were subjected to restraint of various lengths of time In the experiments of longer duration ulcers developed whereas in the shorter experiments no ulcers were seen The aminosugar content of acid polysaccharides in the gastric wall of the glandular stomach were increased slightly in the "pre-ulcerous" groups and fell sharply to below control values in the ulcer groups Fractionation of polysaccharides from
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The restraint method is an effective psychological method for producing ulcers in rat (Bonfils 1964) The ulcers are mostly located in the glandular stomach The time required to produce the ulcers is very short, about 24 hrs although occasionally even less It is thus shorter than when pharmacological agents are used for the same purpose In another paper we have studied the changes in the content of acid polysaccharides in the gastric wall by exogenous introduction of ulcerogenic agents histamine and cinchophen (Hakkinen 1961 Hartiala and Hakkinen 1965) The present study was undertaken in order to find whether purely endogenous factor such as they are present in the restraint ulcer can also produce similar changes as found in the previous studies.

In another paper (Hartiala and Hakkinen 1965, Hakkinen Hartiala and Terho, 1965) we noted that the so-called polysaccharide fractions 1 0 N and 1 2 N resembled the chondroitin sulfuric acid B and that the 0 3 N fraction contained a polysaccharide similar to the hyaluronic acid The 0 5 N 0 6 N and 0 8 N fractions consist of sulfopolysaccharides found in the gastric mucus The same fractionation procedure has been employed and need therefore not be described here again The salt normalities with which these are eluted are used to name the individual fractions as in the previous cinchophen work

TABLE I Groups of "Restraint rats". Macroscopic analysis of the distal stomach:
1 No visible changes
2 Small superficial petechiae or erosions
3 Deep round ulcerations with dark colored blood at the base

Group	1	2	3
Control	10		
5 h restraint		10	
6 h		10	
8 h		10	
12 h.	2	8	
14 h.	3	7	
16 h	1	9	
18 h.	3	7	
20 h	3	7	
24 h.			9
28 h.	4		6
48 h.	1	6	2

Materials and methods

In this experiment 120 female Wistar rats weighing from 180 to 200 g were used. The [illegible] were divided into twelve groups, 10 rats each accord[illegible] Both the control and restrained rats w[illegible] that of Bonfils (1964 For practical re[illegible] 5 6 8 24 28 abd 48 hr groups The res[illegible] in the afternoon During the restraint pe[illegible] the animal the stomach was opened an[illegible] part of the stomach the glandular part [illegible] is sample was homogenised all the layers together Fractionation of acid polysaccharides was performed in a control group and in the 24 hr restraint group with the same method as in our cincophen study The polysaccharides fractionated in this way from the gastric tissue of dog and from the human gastric juice have been characterized elsewhere The results were given in µg aminosugar per g tissue wet weight. In addition to this the aminosugar content of unfractionated acid polysaccharides were analysed for each group

Results

Only one rat in each of the groups of 24 and 48 hrs died for unknown reasons

Macroscopic lesions in the stomach The lesions were seen to be localised in the glandular stomach No lesions were seen in the squameous-epithelium of the fore-stomach or in the duodenum The stomachs were divided into 3 groups according to the nature of the lesions produced 1 intact stomach, 2 small petechias or quite superficial erosions 3 round ulcer, one or more with dark blood at the base of the ulcer (Table I) After a restraint period of 5 to 20 hours no ulcers were observed only smaller lesions of the second category in the above classification These were more abundant and defined at the beginning of the restraint i e 5—8 hrs (the restraint for these groups was begun in the morning, which may play a part in this finding Most of the ulcers (group 3) were found in the 24-hr group

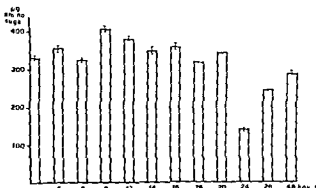


Fig 1 Histogram showing the effect of restraint time on the total acid polysaccharides of the distal gastric wall in rat. The corresponding values are expressed as µg aminosugar/g tissue wet wt.

Total acid polysaccharides in the glandular stomach

In Fig 1 the average amount of polysaccharides in every group is shown, expressed as µg aminosugar per gram gastric tissue wet weight and the standard error After a short restraint (8 hrs duration) there was some tendency for the acid polysaccharides to be increased After restraint of 14 to 20 hrs there was no significant difference from the control group The 24-hr group showed a large decrease and that is why this group was chosen for the fractionation analyses The values for the 28 and 48 hr groups are lower than in the control group also, although not so low as those for 24 hrs

The fractionation analyses of acid polysaccharides

In Table II the amount of aminosugar as µg per g tissue wet weight are shown for the polysaccharide fractions — expressed according to the salt concentrations in the stepwise elution as described elsewhere It can be seen that the fractions 0 3 N 0 8 N, 1 0 N and 1 2 N are highly significantly smaller in the 24-hr group compared to the control group

TABLE II Results of fractionation from two groups of rats the controls and the 24 hr restraint group The individual acid polysaccharide fractions are expressed as µg aminosugar/g gastric wall wet wt The fractions are characterized by the corresponding salt elution concentrations (for details see text)

Fraction	Control rats	24 hr restraint rats	Significance of difference
0 3	103 4 ± 4	79 0 ± 3 4	P < 0 0005
0 5	58 8 ± 2 4	59 6 ± 6 4	0 47 P > 0 45
0 6	28 0 ± 3 4	23 0 ± 4 8	0 25 > P > 0 20
0 8	33 8 ± 2	17 8 ± 1 8	P < 0 0005
1 0	42 2 ± 5 2	10 4 ± 3	P < 0 0005
1 2	31 6 ± 2 4	6 4 ± 2	P < 0 0005

Discussion

From this work we cannot say if the polysaccharides in the gastric wall of the rat are similar to those of dog. But comparing the changes in the dog after cinchophen feeding with the changes in the rat after a restraint period of 24 hrs we note these changes to be quite similar; also the fractionation (Table II) is similar. For this reason there may be a similarity between the gastric polysaccharides in these two species.

This experiment shows that mechanisms in the organism itself can produce changes in the content of acid polysaccharides of the gastric wall in rat similar to changes produced by cinchophen feeding in the gastric wall of dog. A result analogous to this is noted in guinea pig after injection of histamine (Häkkinen 1961).

The 24 hr group showed the worst ulcer lesions and the diminution of acid polysaccharides was greatest in this group. We also note that the amounts of the polysaccharides in non ulcer and ulcer rats did not differ in the 28 and 48 hr groups so the diminution of the polysaccharides could be pre ulcerous.

This study has been supported by grant AM-6018 National Institutes of Health, United States Public Health Service.

References

[illegible]
acid polysaccharides of the gastric wall in dog. *Acta chem. scand.* 1965. *19.* 800—806.

Häkkinen, I., K. Hartiala and T. Terho: The fractionation and characterisation of acid polysaccharides in human gastric juice. *Acta chem. scand.* 1965. *19.* 797—799.

Häkkinen, I.: Histamine induced changes in the sulphate metabolism of the duodenum and prepyloric region of the stomach: an experimental study with special reference to ulcer formation. *Acta physiol. scand.* 1961. *51.* Suppl. 177.

Acta physiol. scand. 1966. 66. 337—345

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Blood Flow through Human Adipose Tissue Determined with Radioactive Xenon

By

O Andrée Larsen, N. A Lassen and F. Quaade

Received 20 August 1965

Abstract

Larsen, O A , N A Lassen and F Quaade. *Blood flow through human adipose tissue determined with*

Measurement of blood flow in a tissue on the basis of the observation of the clearance rate of a radioactive isotope injected directly into that tissue was introduced by Kety in 1948 Kety studied muscle blood flow using Na^{24} as the isotope and recently Kety's principle has been applied to the measurement of blood flow in skeletal muscles using instead, radioactive Xenon[133] dissolved in saline (Lassen *et al* 1963, Lassen, Lindbjerg and Munck 1964, Holzman *et al* 1964) A radioactive inert gas such as Xe^{133} is probably a better indicator of blood flow than Na^{24}, because Xenon, due to its high lipid solubility, can be assumed to cross the lipidcontaining cellular membranes freely (Lassen 1964)

In the present study the local clearance of Xe^{133} has been applied to blood flow measurements in the adipose tissue in human subjects

Method

Xenon 133 dissolved in sterile isotonic saline solution in an initial concentration of about 1 0 mC per ml was obtained from the Radiochemical Center Amersham, England In order to minimize vasomotor reactions the solution contained no bacteriostatic preservatives

1—3 injections of 0 1 ml each were made slowly each injection lasting at least 30 sec The number of injections depended on the radioactivity in the solution The needle used

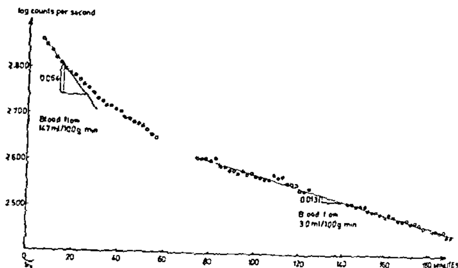


Fig. 1. Xe[133]-clearance curve in a 34 year-old woman with an adipose layer of the abdomen of 32 mm. The count ng was ... min. It was ... the most pro... curve is 0.00... tively.

outer diameter of 0.4 mm and the injections were made a little below and lateral to the umbilicus and at a depth corresponding to the middle of the subcutaneous fat. Great care was taken not to inject any gas bubbles, however small, and in order to minimize reflux of Xe[133] along the needle tract, the needle was not withdrawn from the fatty tissue until half a minute after the injection.

The thickness of the subcutaneous layer was measured in an adjacent area with a needle inserted vertically to the fascia of the abdominal muscles. Comparison of results from ultrasound and needle puncture measurements in 13 patients with an abdominal fatty tissue layer between 6 and 40 mm showed a correlation coefficient of 0.984 (Bullen *et al.* 1965).

The investigation was performed at room temperature and the disappearance rate of the isotope was measured with a sodium iodide (Tl) crystal having a diameter of 5 cm and covered by a cylindrical collimator of 1/2 cm lead. The distance from the crystal to the collimator opening was about 10 cm and the crystal was placed about 20 cm from the skin, covering an area with a diameter of about 15 cm. The counts were registered in 100 sec intervals every second minute for 1—3 hrs after injection. By using a spectrometer, only the photopeak of 81 Kev was measured and the initial counting rate ranged from 5 × 10⁴ to 10⁵ per minute.

The patient was told not to make the smallest movement during the counting procedure and he was under constant observation, so only the respiratory movements of the abdominal wall could change the geometry.

Calculations

Fundamental to the calculations is the assumption of maintenance of complete or almost complete diffusion equilibrium, i.e. that at all times during the study

$$C = \lambda \cdot C_{blood} \quad (1)$$

where C is the amount of Xe[133] ... of Xe[133] per ml of ... been ... lated ... 0.1)

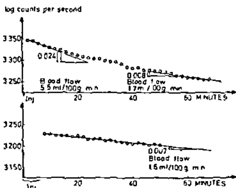


Fig 2 Two Xe[133]-clearance curves from a 40-year-old woman with an adipose layer on the abdomen of 70 mm In the upper half of the figure 3 × 0 1 m Xenon solution was injected over 10 sec and in the lower half 3 × 0 1 ml Xenon solution was injected over 6 minutes Only in the first case a rapid initial clearance rate is seen

$\frac{\text{cpm per g fat}}{\text{cpm per ml blood}}$ · λ varies somewhat with the hematocrit value (Conn 1961), but as our subjects had hematocrit values within the normal range, no correction has been made for this variation.

Application of the Fick principle and insertion of equation (1) leads to equation (2) as the concentration of Xe[133] in the arterial inflow is negligible (no recirculation)

$$dC/dt = -(f/\lambda)\ C \tag{2}$$

where dC/dt is the loss per minute of Xe[133] per g tissue, and f is the blood flow in ml per g/min Solving equation (2) for C one obtains Kety's classical mono-exponential clearance function, viz C(t) = C(O) exp (— (f/λ) t) Equation (2) may also be solved directly for f

$$f = -\lambda\ \frac{dC/dt}{C} = -\lambda\ d\ln C/dt \tag{3}$$

The fatty blood flow per 100 g of fatty tissue is F B F = 100 f Since lnC = ln 10 log C = = 2 3 log C it follows that

$$F.B\ F = 2{,}300\ \lambda\ d \text{ in ml/100 g min.} \tag{4}$$

where d = d log C/dt is the numerical value of the slope to the tangent of the curve of log C plotted against time This way of handling equation (2) is practical at very slow clearance rates (see Fig 1 and 2)

Results

Methodological studies With the present technique the depot is extended over a volume of about 1 × 1 × 1 cm as demonstrated by autoradiography, i e a small area compared to that viewed by the crystal with the collimator used That isoefficiency is adequately maintained despite the softness of the gamma radiation (81 Kev) was shown experimentally a constant counting rate was observed after injection of Xe[133] into the abdominal fatty tissue immediately post mortem, i e no measurable amount of tracer is lost by diffusion from the volume seen by the scintillation crystal

In all cases examined the disappearance rate of the isotope was more rapid in the first 15—60 min after the injection (Fig 1) The inital rapid clearance rate was usually about 3—4 times as fast as that of the rest of the curve and it was most pronounced in subjects with a thin subcutaneous layer If the isotope-containing saline was injected very slowly (about 1 2 min for each injection the initial phase was practically absent or at least much less pronounced than when injecting over 10 seconds (Fig 2)

Two main factors appeared to contribute to this phase of the curve, viz local hyperemia due to the trauma of the injection and reflux of the istope to the dermal tissues with

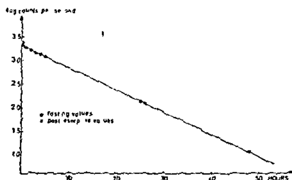


Fig 3 Xe[133]-clearance curve in a 51-year-old man with an adipose layer on the abdomen of 23 mm The injections were made over 4 min and the counting started immediately after and continued for 4 hrs After a rest of 1/2 hr where the patient had dinner, the counting was continued for a further 2 1/2 hrs The counting was resumed 25, 28 and 48 hrs after the injection, each time for about 1 hr It is seen that the postresorptive values the first day indicate a faster clearance whereas the mean clearance rate throughout the 48 hrs shows a monoexponential curve

higher clearance rates The role of tissue trauma was suggested by the finding of an initial phase of relatively fast clearance, even in studies employing a very long injection needle thus avoiding reflux That reflux to the skin can occur when the depote was placed 2—3 mm under the skin using a short needle was demonstrated autoradiographically[1] It is likely that both factors mentioned become less important when employing the slow injection technique described above

However, it has not been possible to find a procedure which completely abolishes the initial phase, and therefore in all studies referred to below the Xe[133] clearance from fatty tissue was first recorded from at least 60 min after the injection and the measurement was continued for the next 60 min or more

The slope of the clearance curve is fairly constant after the initial phase This was found both in several experiments where the slope was registered continuously for 2—4 hours after injection (Fig 1), and in some experiments where the clearance curve was registered repeatedly through 48 hrs after the injection As seen in Fig 3 an approximately mono-exponential decrease of the isotope concentration was found after the first few per cent of the dose had been cleared an until the end of the observation, when 99 per cent had been cleared Thus this simple function describes the overall clearance fairly well even though differences of exponential coefficient may occur over shorter time intervals

As maintenance of diffusion equilibrium between tissue and blood is essential for the method used, experiments were performed in which the relative disappearance rate, $k = -C'/C$, of both Krypton (Kr^{85}) and Xenon (Xe^{133}) injected into the adipose tissue in the same subject were measured (table I) If blood flow is the rate limiting factor, then one obtains from equation (2) that $k_{Kr}/k_{Xe} = \lambda_{Xe}/\lambda_{Kr}$ If on the other ... rate

constants, D

The ratio λ_{Xe} λ_{Kr} can be estimated to be 1 75 from the ratio of their olive oil water partition coefficients (expressed Xe Kr, this ratio is 20 0 9 6 = 2 08 (Lawrence *et al* 1946)), corrected by multiplying it with the ratio of their water air partition

[1] The autoradiography was kindly performed by dr P Sejrsen.

TABLE I Disappearance rates, $k = -C'/C$ in min^{-1}, of Krypton-85 and Xenon 133 determined simultaneously after injection into the abdominal adipose tissue in the same patient

Case		k_{Kr}	k_{Xe}	k_{Kr}/k_{Xe}
49	I H	0 0120	0 0061	1 96
50	E O	0 0019	0 0009	2 10
51	J L	0 0118	0 0064	1 83
52	L.M.	0 0043	0 0021	2 07
54	V M	0 0027	0 0013	2
Mean				2 01
SE of mean				0 06
$\lambda_{Xe}/\lambda_{Kr}$ (determined from their olive oil blood partition coefficients)				1 75
$D_{Kr}/D_{Xe} = \sqrt{M_X} / \sqrt{M_{Kr}}$ (M=molecular weight)				1 25

coefficients (expressed Xe Kr, thus ratio is 0 097 0 051 = 1 90 (Lawrence *et al* 1946) and with the ratio of their air blood partition coefficient (expressed Xe Kr, this ratio is 6 85 15 6 = 0 44 (Mellemgaard, Lassen and Georg 1962, Veall 1965) The ratio D_{Kr} D_{Xe} can be estimated to be 1 25 as this is the square root of the inverse ratio of the respective molecular weights of the two gases The average value for the ratio k_{Kr} k_{Xe} is 2 01, SE of mean 0 06 Being even above the estimated λ ratio, this value is consistent with the concept that the molecular size is unimportant relative to solubility

Blood flow in abdominal adipose tissue The study comprised 69 examinations in 55 ambulatory patients (17 men and 38 women) suffering from minor diseases (neurosis, gastric ulcer, varicose veins) With regard to the general health condition these subjects were normals They were selected to represent different states of nutrition, ranging from normal weight to pronounced obesity Patients with a subcutaneous layer below 10 mm have not been included in this normal material Since the average radius of the depot is 5 mm this selection of the subjects secures negligeable reflux of Xe^{133} into the skin The examination took place in the morning the patients being fasting and recumbent 14 of these patients were examined twice with intervals from 2 days to 7 months.

It is seen from table II that the average fatty tissue blood flow for the whole group was 2 6 ml/100 g min There was no significant difference between the fatty blood flow in man and women or between younger and older subjects

Separating the patients in three groups according to the thickness of their abdominal adipose tissue layer the average value of the fatty blood flow in patients with a layer ranging from 10 to 29 mm was 3 0 ml/100 g min in the patients with a layer of 30 to 49 mm it was 2.2 ml/100 g min and in the patients with a layer of 50 to 120 mm it was 2.5 ml/100 g min The difference between the first and second of these groups was statistically significant The last group includes two very obese patients, in whom repeated measurements showed very high blood flow values If they are excluded from

TABLE II

Group	No of subjects	No of studies	FBF ml/100 g min Mean	SD	P
Total	55	69	2 63	1 72	
Men	17	21	2 77	1 77	> 0 30
Women	38	48	2 57	1 68	
Subjects < 40 years	23	29	2 69	1 78	> 0 40
Subjects > 40 years	32	40	2 59	1 70	
Thickness of adipose tissue between 10 and 29 mm	25	30	3 04	2 09	< 0 05
Thickness of adipose tissue between 30 and 49 mm	19	22	2 15	1 22	
Thickness of adipose tissue > 49 mm	11	17	2 53	1 42	< 0 05
Thickness of adipose tissue > 49 mm (—2 subjects see text)	9	13	1 84	0 71	

TABLE III Blood flow values determined simultaneously with Xe^{133} in subcutaneous fatty tissue at different sites of the body

Case		Abdominal wall FBF ml/100 g min	Abdominal wall Thickness of adipose tissue in mm	Gluteal region FBF ml/100 g min	Gluteal region Thickness of adipose tissue in mm	Front of the thigh FBF ml/100 g min	Front of the thigh Thickness of adipose tissue in mm
48	S J	2 1	37 mm	2 2	10 mm		
53	A M	2 6	33 mm	3 3	22 mm		
55	K J	5 4	14 mm			3 6	11 mm
56	T J	1 5	32 mm			2 0	20 mm
57	E M	0 9	75 mm			0 9	43 mm

the group the average flow value is 1 8 ml/100 g min and the difference between all 3 groups is significant

The regression equation for all the subjects studied (y = flow in ml/100 g min, x = thickness of fatty tissue in mm) is $y = -0\ 0314\,x + 3\ 08$ with a correlation coefficient of 0 17 ($p < 0\ 05$) and excluding the 2 patients mentioned above gives a regression equation $y = -0\ 0411\,x + 3\ 95$ with a correlation coefficient of 0 49 ($p < 0\ 001$)

Considering the studies as duplicate measurements the coefficient of variation of F B F was 25 per cent a value including the possible biological variations

Blood flow in other parts of the subcutaneous tissue For reason of comparison observations were made in other parts of the subcutaneous fat It will be seen (table III) that the blood flow values measured simultaneous in various localizations (abdomen hip front of thigh) in the same person were in the same range, also with a tendency to higher values for a lesser thickness of fatty tissue

Discussion

The present method is based on the principles discussed extensively by Kety in 1951 The area injected is presumed to be homogenous with respect to blood flow and inert gas solubility Also, maintenance of diffusion equilibrium is assumed

Tissue homogeneity is supported by the agreement of the observed Xe^{133} clearance to the theoretical mono-compartmental model even in the very prolonged studies carried on until only about one per cent of the injected isotope remained in the depot A curve oscillating around a simple mono-exponential function was obtained i e this function describes the average clearance very well over no less than five time constants For an exponentially decreasing time function it is convenient to describe the rate of decay be the time constant, i e the time for the concentration to decrease to $1/e$ 0 37 of its original value That diffusion equilibrium is essentially reached is indicated by the theoretical calculations of diffusion in a tissue cylinder made by Copperman and quoted by Kety (1951) Provided this condition prevails, then local redistribution of isotope between adjacent tissue cylinders is of no importance in a homogenous tissue, as demonstrated mathematically by Perl (1962 1963)

Experimental support for maintenance of diffusion equilibrium between fatty tissue and blood was obtained in the double tracer experiments given in table I and discussed above

The solubility coefficient λ was determined by Conn in 1961 by in vitro experiments on fatty tissue and blood from dogs In his studies when the tissue was allowed to equilibrate with the Xe^{133} gas mixture for a period varying from 1 to 8 hrs the λ value was $7.5 \frac{\text{cpm per g fat}}{\text{cpm per ml blood}}$ for a hematocrit of 40 per cent The λ used by us was recently determined by Munck *et al* in vivo studies on rats The animals were equilibrated with the Xe^{133}-gas mixture for 24 hrs (120 samples from 20 rats) and the average figure was 9.8 ± 1.1 expressed in $\frac{\text{cpm per g fat}}{\text{cpm per g blood}}$ for a hematocrit of 50 per per cent or 10.0 ± 1.1 when calculated in $\frac{\text{cpm per g fat}}{\text{cpm per ml blood}}$ for a hematocrit of 40 per cent The longer equilibration time in this study is supposedly the cause of the higher and hence presumably more correct value

[illegible] 1955)

In other parts of the subcutaneous panniculus the fat content probably varies On such sites the use of an indicator with a λ independent of the fat content is essential Preliminary studies suggests that radioiodinated antipyrine has this property (Munck *et al* 1965) However the use of this indicator may give rise to other problems due to radiolysis, slowness of diffusion and the possibility of biological effects

Comparison with other estimates of fatty tissue blood flow The average value of 2 6 ml/100 g min found by us for the blood flow through adipose tissue in the abdominal wall is of the same magnitude as the average flow values for the adipose tissue in the total human body found by Perl, Lesser and Steele (1960) Assuming that the body was composed mainly of three compartments, these investigators made an analysis of the time course of the uptake over 8 hours of the highly fat soluble, inert gas cyclopropane in 6 subjects of normal weight The estimated blood flow to the compartment supposed to represent adipose tissue (the slowest mono-exponentially saturating phase) was in average 3 1 ml/100 g min The errors inherent in this multicompartmental approach has recently been emphasized by the same group of investigators and the value here given must consequently be taken to constitute a fairly rough estimate of F B F (Rackow *et al* 1965)

Recently, an attempt at direct measurement of the blood flow through adipose tissue was made by Oro Wallenberg and Rosell (1965) In 5 dogs they isolated a part of the subcutaneous adipose tissue in the lower abdominal region leaving the vessels supplying the fatty tissue undamaged By cannulating the vein the blood flow through the area was measured directly and found to vary between 3 4 and 9 3 ml/100 g min (mean value = 6 7 ml)

The tendency to a decrease in F B F with increasing thickness of the adipose tissue was an interesting and somewhat unexpected finding It might be due to the influence of the vascularization of the skin, as the somewhat faster clearance rates often found after subcutaneous injections in lean subjects were considered to be due to the proximity of the more highly vascularized dermal tissues or to a lower fat content here (Perl *et al* 1965) However, in our series we have omitted all subjects with a subcutaneous layer below 10 mm hereby practically eliminating this possibility according to the autoradiographic studies Moreover, injections made in the middle of the subcutaneous fatty layer and deep in this layer (just above the fascia) gave the same clearance rate

A possible explanation of the finding is, that the lower blood flow in obesity is a reflection of a lower metabolic demand e g secondary to a reduction in the mass of metabolically active constituents per g tissue This hypothesis is supported by the known swelling of the fat globules of the cells in obesity

The present study aims primarily at describing a method for measurement of the fatty tissue blood flow in man but several interesting physiologic findings have been made Two very obese patients represented an exception to the tendency of decreasing F B F with increasing obesity as repeated measurements showed high blood flow values This isolated observation may be interpreted as a confirmation of the suggestion that there are perhaps several types of obesity Also it has been found that the F B F increases twice or more after administration of proteins perorally and aminoacids intravenously Studies concerning these findings are under development

References

reference to size and number of
ct ons used to determine
Xenon hemoglob n asso-

HOLZMAN, G B, H N WAGNER JR, M IIO, D RABINOWITZ and K. L. ZIERLER, Measurement of muscle blood flow in the human forearm with radioactive Krypton and Xenon *Circulation* 1964 *30* 27—43

[illegible]

In preparation

[illegible]

PERL, W, An extension of the diffusion equation to include clearance by capillary blood flow *Ann N Y. Acad Sci* 1963 *108* 92—105

PERL, W [illegible] gas [illegible]

PERL, W [illegible] effect [illegible]

RACKOW, H, E SALANITRE, R M EPSTEIN, G L WOLF and W PERL, Simultaneous uptake of N_2O and cyclopropane in man as a test of compartment model *J appl Physiol* 1965 *20* 611—620

VEALL, N, Pers comm

Acta physiol. scand. 1966. 66. 346—352

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Excretion of Methylmalonate and Succinate in the Urine of Rats during Deficiency of Vitamin B_{12} and after Administration of Propionate

By

Jussi Asunta, Martti Koivusalo and Markku Suorsa

Received 7 September 1965

Abstract

[illegible] was given already to the pregnant rats and continued to the offspring for 7 weeks the excr[illegible] of methylmalonate by the litters increased 50—100 fold as compared with the corresponding controls If the diet was started later only a slight increase in the excretion of methylmalonate was found When normal rats were fed 1 g of sodium propionate per day for 2 days there was a slight increase in the excretion of methylmalonate and a marked increase in the excretion of succinate Methylmalonate and succinate were isolated and determined by silicic acid column and thin layer chromatography and by paper chromatography

The occurrence of methylmalonate in the urine was first shown by Boyland and Levi in 1936 when they isolated it from rat urine after administration of anthracene Later it was found to be a normal constituent of both rat and human urine (Thomas and Stalder 1957, 1958 Thomas et al 1957) An increase in the excretion of methylmalonate by the rat was found also after experimental liver injury (Forbes et al 1953 Barness Moeksi and Gyorgy 1956 The study of the excretion of methylmalonate in the urine became more interesting after Flavin and Ochoa showed in 1955 that methylmalonate is a normal intermediate in the metabolism of propionate in the animal organism and [illegible]

[illegible] vitamin B_{12}, and methylmalonate would accumulate in the deficient [illegible] experiments reported in the present paper the excretion of methylmalonate and succinate in the urine of rats has been investigated during experimental deficiency in vitamin B_{12} and after administration of relatively large amounts of propionate

Material

Albino rats of the Wistar strain were used in the experiments Group I consisted of rats that were kept deficient in vitamin B_{12} from the embryonic period on For this purpose 8 female rats at the age of 6 5 months were kept on vitamin B_{12} deficient diet (Vitamin B_{12} Deficient Diet from Nutritional Biochemicals Corp , Cleveland 28 Ohio) The pregnancy was initiated after 7 days on the diet. Only the B_{12}-deficient diet was given to the litter Urine was collected when the young were 5 and 7 weeks old and it was collected the same way from control rats of the same age The newborn rats on the B_{12}-deficient diet were much smaller than the controls and died before 2 months of age Urine was collected also from the female rats after 3 months on the diet (Group II) Eight female rats were similarly given the B_{12}-deficient diet at the age of 3 months and the pregnancy was initiated after 7 days These rats however did not bring forth young whereas the control group of the same age brought forth normally Urine was collected also from these rats after being on the diet for 2 months Further the B_{12}-deficient diet was given to normally fed rats at the age of one month (Group III) The urine was collected after 10 17 and 24 days on the diet and similarly from the control rats A significant difference in weight developed between the controls and the rats on the deficiency diet and the deficient rats died at about 2 months of age

The excretion of methylmalonate and succinate was also studied after feeding the rats with sodium propionate in amounts of 0 5—2 0 g per rat daily (Group IV) It was added to the stock food mixture given to the rats The rats of this group were 3 to 8 months old and the propionate diet was started 2 days before collecting the urine The urine was collected in the same way from the controls

Methods

Standards and reagents Methylmalonic acid (Fluka purum) succinic acid (Schering Kahlbaum p a), ether (Merck p a) benzene (Merck crystallisabile p a) methanol Merck p a) ethanol (Oy Alkoholiliike Ab Aa 99 5 % w/w) ammonia (Merck 25 % p a) isopropylether (Baker), isoamylformiate (Fluka purum) silicic acid (Mallinckrodt 100 mesh Kieselgel G nach Stahl (Merck) and Kieselgur G nach Stahl (Merck)

In groups I III and IV the night urine of 15 hours was collected from 12 rats on two consecutive days In group II the night urine was collected from 6 animals on 4 consecutive days In each group the urine was pooled and preserved at 4° C with toluene

For the analysis of the urine samples a method based mainly on that described by Thomas and Stalder (1957, 1958) was used In this method the ether extract of urine is initially purified with strongly basic anion exchange resin and the acidified eluate is extracted with ether for the second time After the acids which are soluble in petroleum ether have been separated the acid mixture obtained is fractionated in a silicic acid column using an ether benzene mixture with increasing concentrations of ether The acid peaks are determined by titration with potassium hydroxide

In our procedure we used a Dowex 2 × 8 (50 mesh anion exchange resin instead of Lewatit MN The resin was first converted to OH form by 10 per cent sodium hydroxide The ether extract of the urine sample was run through the column which was then washed with 1 5 liter destilled water and eluted with 300 ml of 1 N NH_4Cl The ether extract obtained from the acidified eluate (pH 1) was boiled thoroughly with petroleum ether (AnalaR B P 40 -60 C) which was drained out afterwards and discarded The mass left was dissolved in absolute ether and was absorbed into 0 5 g silicic acid for column chromatography The chromatography was done in a silicic acid column (9 5 g using 0 5 N sulphuric acid as stationary phase and increasing concentrations of ether (10 % 20 % 30 % in benzene as mobile phase The dry silicic acid powder containing the raw acid mixture was transferred quantitatively to the top of the column and was washed with 40 ml of 10 per cent ether benzene mixture after which the collection of fractions was started Methylmalonic and succinic acids were first separated from other acids. When pure standards of methylmalonic and succinic acids were used and fractions of 3 ml collected methylmalonic acid began to flow out of the column in the fraction number 115 and when 250 fractions were collected succinic acid had come quantitatively out from the column. Thus fractions 110—255 were collected and divided into two equal portions However fractions 110—270 were collected in the propionate administration experiments to

be sure of quantitative recovery of succinic acid and in every analysis also the fractions 95—110 were preserved [illegible]

peaks were identified with thin layer chromatography using 3 different methods 1) Kieselgul G plate and benzene-methanol-acetic acid (48 : 8 1, v/v/v) as solvent A saturated chamber [illegible]

in the latter half of complex I of the titration curves In the curves obtained when analyzing the urine of the rats onto B_{12}-vitamin deficient diet after birth (Group III) it was possible to see a separate peak in the latter half of complex I, which according to TLC contained mainly methylmalonic acid In the urine of rats that already in the embryonic period were vitamin-B_{12}-deficient (Group I) it was found that peak I contains on the basis of TLC, only methylmalo- [illegible]

graphy

The second half of the eluate obtained from the first column chromatography and containing the methylmalonic and succinic acids was evaporated to dryness and dissolved in acetone Methylmalonic and succinic acids were determined quantitatively using two-dimensional ascending paper chromatography (Barness *et al* 1963) The solvent mixtures used were 1) Ethanol ammonia water (200 9 4, v/v/v) The Rf value of methylmalonic acid was 0 16 and [illegible] (220 40 20, v/v/v) The [illegible] ral different [illegible] to methyl-malonic and succinic acids were cut out extracted with warm (80°) distilled water and titrated with 0 001 N potassium hydroxide using o-cresol red as indicator For the determination of [illegible]

Results

The results are presented in Fig 1—4

The excretion of methylmalonate in the urine of the rats on B_{12}-vitamin deficient diet already during the embryonic period (Group I) was about 50—100 times higher [illegible]

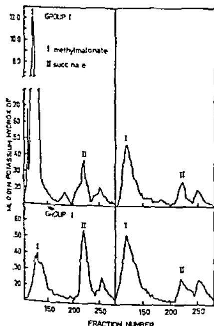


Fig 1

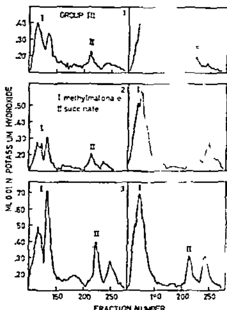


Fig 2

Fig 1 S…

Fig 2 Se… pooled rat urine by silicic acid … e age of … urve 3) … ght were

diet after birth (Group III) show that after a diet for 24 days there is a somewhat higher excretion of methylmalonate in the urine than that of the controls, whereas the excretion of methylmalonate was not higher in the urine of rats kept on the diet for 10 or 17 days than in the controls It should be noted that the rats on vitamin B_{12}-deficient diet were much smaller than the controls and that their mortality was considerable already two weeks after beginning of the diet The titration curves obtained from the urine of these rats show a small response in the excretion of methylmalonate already after 10 days' diet, similarly the ratio methylmalonate succinate is higher than that of the controls There also was no marked difference in the excretion of succinate when compared to the controls

The female rats of Group II showed on the contrary no difference in the excretion of methylmalonate when compared with the controls despite the fact they had been on vitamin B_{12}-deficient diet for two moths and that those aged 8 5 months had gone

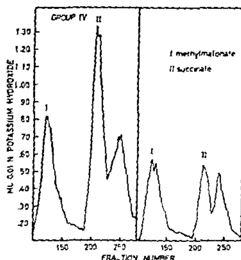


Fig 3

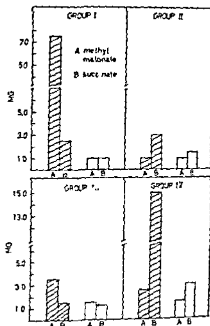


Fig 4

The curve on the right was obtained from the urine of the controls

Fig 4 The amounts of methylmalonic and succinic acids in pooled rat urine determined by

propionate per rat and per day during 2 days age 7 months The urine was collected ... 30 hrs and half of it was analyzed Lined columns Test rats Blank columns Controls

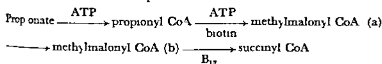
through a pregnancy with a delivery and those aged 5 months had aborted early in the pregnancy

In the experiments where propionate was administered to the rats (Group IV) it was not possible to show clear changes in the excretion of methylmalonate and succinate when the dose was 0 5 g sodium propionate per day When 1 g or 2 g of propionate was given daily there was a distinct increase in the excretion of succinate and only a slight increase in the excretion of methylmalonate

According to our results the excretion of methylmalonate by a normal rat is about 13—20 µg per day the excretion by an adult rat being slightly higher than that by a ... The excretion of succinate at age of 1 to 2 months a approximately the same ...

Discussion

According to the present concepts of the metabolic pathway of propionate in the animal organism (Kaziro and Ochoa 1964) propionate is first activated to propionyl CoA which is then carboxylated in an ATP requiring reaction to methylmalonyl CoA. The methylmalonyl CoA formed is then racemized to its optical enantiomorph and the product is isomerized to succinyl CoA by methylmalonyl CoA mutase (E.C. 5.4.99.2, 2-methylmalonyl CoA — CoA carbonyl mutase). A coenzyme form of vitamin B_{12} is required for this isomerization reaction. The pathway of propionate metabolism in the animal tissues can be presented as follows



The present results show that in the rat the methylmalonyl CoA pathway of propionate metabolism is utilized rather effectively and that large amounts of methylmalonate are excreted in the urine of vitamin B_{12}-deficient animals contrary to the very low excretion by the normal animals. This increase in the excretion of methylmalonate is clearly due to a block in the methylmalonyl CoA mutase step in the metabolism of propionate in these animals.

Smith and Monty (1959) have earlier shown a marked decrease in the methylmalonyl CoA mutase activity of livers from vitamin B_{12} deficient rats as compared to those from normal rats, and Gurnani, Mistry and Johnson (1960) demonstrated that the dimethyl benzimidazolyl cobamide coenzyme restored the mutase activity of liver extracts from deficient animals. It has also been observed in human patients with pernicious anemia that the excretion of methylmalonate in the urine is greatly increased and decreases during treatment with vitamin B_{12} (White 1962, Barness *et al.* 1963, Asunta and Suorsa, to be published).

The administration of relatively large amounts of propionate did not increase the amount of methylmalonate in the urine of normal animals significantly but caused a clear elevation in the excretion of succinate. This also indicates that the methylmalonyl CoA mutase reaction is not a limiting step in the propionate metabolism in the normal rat.

When the excretion of methylmalonate and succinate in the urine of rats on the normal stock diet was examined at different ages there was some decrease in the ratio of methylmalonate to succinate in the older animals. Growing rats on vitamin B_{12}-deficient diet began also to excrete larger quantities of methylmalonate after less than one month on the diet, while a full grown female had no increase in the excretion of methylmalonate even during a simultaneous pregnancy.

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References

BARNESS, L. A., H. MOREY and P. GYÖRGY, Urinary excretion of methylmalonic, α, α-dimethylsuccinic and other ethersoluble acids in rats. *J. biol. Chem.* 1956 221 93—100.

BARNESS, L. A., D. YOUNG, W. J. MELLMAN, S. B. KAHN and W. J. WILLIAMS, Methylmalonate excretion in a patient with pernicious anemia. *New Engl. J. Med.* 1963 257 144—14[illegible].

BOYLAND, E. and A. A. LEVI, The isolation of methylmalonic acid from rat urine. *Biochem. J.* 1953 39 2007—2008.

FLAVIN, M. and S. OCHOA, Metabolism of propionic acid in animal tissues. I. Enzymatic conversion of propionate to succinate. *J. biol. Chem.* 1957 229 965—979.

FORBES, M., L. A. BARNESS, H. MOREY and P. GYÖRGY, Excretion of ether-soluble acids by rats on necrogenic diet with and without supplement of antibiotics. *Proc. Soc. exp. Biol.* (N.Y.) 1953 84 162—165.

GURNANI, S., S. P. MISTRY and B. C. JOHNSON, Function of vitamin B_{12} in methylmalonate metabolism. I. Effect of a cofactor form of B_{12} on the activity of methylmalonyl CoA-isomerase. *Biochim. Biophys. Acta* 1960 38 187—188.

[illegible] and S. OCHOA, The metabolism of propionic acid. *Adv. Enzymol.* 1964 26 283—378.

[illegible] tamin

[illegible] ns Ber

THOMAS, K., H. KALEE, I. NAGAI and K. STALDER, Über das Vorkommen von Methylmalonsäure im Harn. *Hoppe-Seylers Z. physiol. Chem.* 1957 308 213—219.

THOMAS, K. and K. STALDER, Isolierung von Methylmalonsäure aus normalem menschlichem Harn. *Chem. Ber.* 1957 90 970—974.

THOMAS, K. and K. STALDER, Über die Herkunft der Methylmalonsäure im Harn. *Hoppe-Seylers Z. physiol. Chem.* 1958 313 22—29.

WHITE, A. M., Vitamin B_{12} deficiency and the excretion of methylmalonic acid by the human. *Biochem. J.* 1962 84 41P.

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Estimation of the Protein Concentration of the Capillary Filtrate by an Isotope Technique

By

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Received 10 September 1965

Abstract

Appelgren [illegible]
[illegible]
the simultaneous measurement of changes of the activity from Cr^{51}-labelled erythrocytes The difficulties and errors of the method are discussed The protein content of the capillary filtrate is low both during venous congestion and exercise, about 0 3 g/100 ml or less than 10 % of that of plasma

As a control of the method the protein content of the filtrate formed during continuous intra arterial infusion of histamine was also studied In these experiments the protein concentration of the capillary filtrate rose to as much as 75 % of that in plasma

The protein concentration of the capillary filtrate is one determinant of the trans-capillary gradient of colloid osmotic pressure and gives an estimate of the permeability of the capillary membrane

Attempts have been made to estimate the protein concentration of the capillary filtrate from the composition of lymph or edema fluid, but neither of these fluids is identical with the capillary filtrate

Landis *et al* (1932) measured the differences of hematocrit and plasma protein concentration in the venous blood from congested and uncongested human forearms When the venous pressure was raised to 60 mm Hg the protein concentration of the capillary filtrate was estimated to 0 3 g/100 ml With a congestive pressure of 80 mm Hg the protein concentration rose to 1 8 g 100 ml By a similar technique Halpern *et al* (1955) arrived at values of 1 9—3 2 g/100 ml filtrate using a congesting pressure of 80 mm Hg Szabo *et al* (1963) used a similar technique in canine limbs and found values

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between 0.8 g/100 ml at low congesting pressures and 3.2 g/100 ml at pressures above 60 mm Hg.

Pappenheimer and Soto-Rivera (1948) found in severely dehydrated tissues that the isogravimetric capillary pressure decreased below the plasma protein osmotic pressure. This was ascribed to an increase of the protein concentration in the tissue fluid. By measuring the amount of capillary filtrate needed to restore the isogravimetric pressure to that of the plasma colloids, they estimated the protein concentration of the filtrate to 0.2—0.4 g/100 ml.

The present study was prompted by our previous finding (Jacobsson and Kjellmer 1964) that lymph flow from exercising calf muscles in cats corresponded only to a minor fraction of the total capillary filtrate formed during the exercise. Since the muscle lymph had a high protein content, all proteins of the capillary filtrate might return to the blood circulation via lymph if the protein content of capillary filtrate was low. As described above experimentally obtained values for the protein concentration of capillary filtrate during venous congestion vary widely and values during exercise are lacking.

The method to be described uses a plethysmograph to measure volume changes in the calf muscles of cats during venous congestion and muscular exercise. The amount of protein in the capillary filtrate during these procedures was estimated by external monitoring over the muscle of the activity from radioactive iodinated human serum albumin (RISA). To correct for changes in activity due to changes in regional blood volume the emission from erythrocytes tagged with Cr^{51} was continuously measured. As a control of the technique i. a. infusions of histamine were included.

Methods

The experiments were performed on 10 cats in 5 expts., anesthetized with chloralose 70 mg/kg or nembutal 35 mg/kg. During the course of the experiments additional amounts of nembutal were given. Heparin (5 mg/kg) was given at the end of the operative preparation and half that dose repeated every 3—4 hr of the experiment.

Plasma proteins were determined by a modified Biuret method on duplicate samples and electrophoretic separation was made according to Laurell (Laurell and Skoog 1956). The injected RISA was localized by a paper strip scanner and the activity was found to travel electrophoretically with cat albumin.

The hematocrit was measured with a capillary centrifuge giving an error of less than 2 %.

To enable the preparation of the hind limb on an isotope free cat and also allow sufficient time for the equilibration of the injected isotopes in the cat, 2 cats were used in each experiment. [illegible] RISA and Cr^{51} tagged [illegible] lood circulation of [illegible] d for equilibration [illegible] scribed by Kjellmer [illegible] skin in the present experiments.

The popliteal artery and vein were cannulated with polyethylene tubing and the femur cut, [illegible] connected to the femoral vessels of cat II via [illegible] saline according to Owen (1959 [illegible] calf preparation and cat II was [illegible] dine less than 1 %) was injected. [illegible] 40—60 000 cpm. [illegible] d plethysmograph were recorded

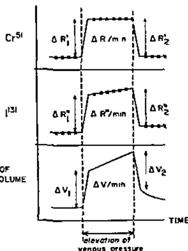


Fig 1
R' = activity from Cr^{51}
R'' = activity from I^{131}
ΔR = change of activity
$\Delta R_{1,2}$ = change of R in 1 and 2 resp
$A = \Delta R'_1/\Delta V_1 = \Delta R'_2/\Delta V_2$
$B = \Delta R''_1/\Delta V_1 = \Delta R''_2/\Delta V_2$
ΔV_f = net filtration/min $= \Delta V - \Delta R'/A$
ΔV_{Cl} = ml plasma cleared from albumin/min $= \left(\Delta R'' - \Delta R' \frac{B}{A}\right) (1 - H)/B$
H = hematocrit

The venous outflow from the calf was measured with a photoelectric drop-recorder operating an ordinate writer The arterial blood pressure was recorded with a mercury manometer from a side tube on the tubing close to the popliteal artery

The peripheral stump of the cut sciatic nerve was placed on bipolar electrodes and stimulated from a Grass stimulator model CR

Gamma rays from Cr^{51} and I^{131} were measured by a scintillation detector with a 1 3/4 × 2 inch NaI crystal placed close to the plethysmograph over the calf Collimation with a lead [illegible] small volume changes The intravascular and interstitial spaces were supposed to have the same measuring geometry in the limb

Standard preparations of Cr^{51} and I^{131} each giving approximately the same activity and geometry of the gammabeam as the calf were used to control the stability of the measuring device before and after each phase of the experiments While measuring the standard preparations the calf preparation was shielded by lead without changing the positions of detector and limb The background from the limb was corrected for

Cr^{51} and I^{131} have gamma ray energy peaks with maxima at 323 and 360 keV resp This means that the two isotopes cannot be measured separately on two channels These isotopes were anyhow considered the most convenient ones for tagging erythrocytes and albumin Pulses from the detector and preamplifier unit were fed into a 2-channel spectrometer (Model Tricarb 314E, Packard) with channel settings at (1) 270—325 keV and (2) 325—420 keV so that activity of channel (1) is due mainly to Cr^{51} and activity of channel (2) mainly to I^{131} The activities on the two channels were registered by two scaler units with 0.5 or 1 min counts and also continuously by 2 ratemeter recorder units (time constant 3 or 10 sec)

The relative activities of Cr^{51} R and of I^{131} R were calculated in the following way

$$R = R_1 - \alpha R_2 \qquad R = R_2 - \beta R_1$$

where R_1 and R_2 are the pulse rates registered on channel (1) and (2) resp α and β are the ratios obtained with the standard preparations

$$\alpha = \frac{(R_{I\text{-stand}})_1}{(R_{I\text{-stand}})_2} \qquad \beta = \frac{(R_{Cr\text{-stand}})_2}{(R_{Cr\text{-stand}})_1}$$

No correction for the radioactive decay of Cr^{51} and I^{131} was necessary

The activities of the two isotopes thus calculated were plotted in a diagram (cf Fig 1 and 2). Linear parts of the curves were evaluated by the method of least squares

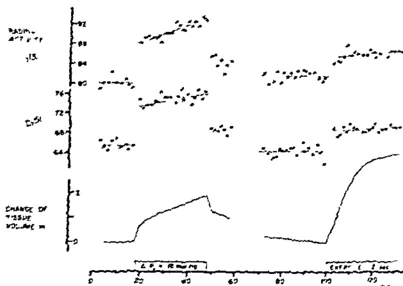


Fig. 2. Exp. 1. Change of activity emitted from I^{131} and Cr^{51} and change of tissue volume during a period of venous congestion followed by a period of muscular contractions.

The activity changes of Cr^{51} and I^{131} and the volume change during a period of venous congestion are shown schematically in Fig. 1. In the figure A = Cr^{51} activity change/ml blood (calculated from the volume curve), B = I^{131}-activity change/ml blood. A and B were calculated at the beginning and at the end of venous congestion, i.e. for rapid increase and decrease of regional [illegible]

[illegible]

I^{131}-acti[illegible]

cnt. gr[illegible]

100 [illegible]

centration in the plasma.

Discussion of methods

The main difficulty of the present method is due to the [illegible] relation between a small amount of protein in the capillary filtrate and a large amount in the regional blood volume. Apart from the unfavourable statistical situation this means that small fluctuations in the regional blood volume must be detected accurately and the I^{131} activity corrected accordingly. This was done by following the activity emitted from the erythrocyte bound Cr^{51}. However, two problems arise with this correction: 1) the separation between I^{131}-activity and Cr^{51}-activity is critical, and 2) it is difficult to evaluate the activities from two pools gradually accumulating in poorly perfused parts of the preparation.

The separation of gamma-energies from I^{131} and Cr^{51} can be performed adequately provided the channel settings on the pulse height analyzer are reasonably stable. Good stability of the channels was achieved (cf. Öbrink and Ulfendahl 1963) by keeping the detector exposed to a high and constant activity of the same order as that to be used in the experiment for several hours before the experiment was started.

To estimate how much activity was accumulated in badly perfused parts of the preparation during the experiment the calf was dissected into muscle, skin and bone at the end of the

TABLE I

		Exp no	Duration min	Filtered volume ml	Albumin conc in plasma g/100 ml	Albumin conc in filtrate	
						% of plasma conc	g/100 ml
Congestion mm Hg	18	1	30	1 3	1.2	10± 3	0 12±0 04
	11	2	13	0.24	1 7	18±20	0 31±0 34
	30	3	23	2 5	2 8	−0 9±2 0	−0 03±0 06
	25	5	44	2 4	3 1	18±14	0 56±0 43
	15	5	26	0 44	3 1	−2±13	−0 06±0 40
Mean					2 4	8 6	0 18
Exercise contractions/sec	2/s	1	30	3 4	1 2	−2± 4	−0 02±0 05
	2/s	2	17	4 3	1 7	0 7±0.2	0 01±0 003
	2/s	3	29	4 7	2 8	−0 7±0.9	−0 02±0 03
	1/s	5	13	1 6	3 1	14±4	0 43±0 12
	2/s	5	19	2 7	2.9	10±4	0 29±0 12
Mean					2 3	4 4	0 14
Histamine μg/min	20	2	20	1 9	1 7	46	0 8
	30	3	20	1 2	2 8	90	2 5
	30	4	13	2 7	2 7	78	2 1
	15	5	45	18 5	2 9	83	2 4
Mean					2 5	74	2 0

[illegible]

implications the net *volume filtered* (ΔV_f) *was somewhat underestimated* and the protein concentration of the filtrate correspondingly overestimated and also that the correction of the I^{131} value due to changes of regional blood volume during the filtration phase is correct only if I^{131} and Cr^{51} accumulated in the skin and bone in the same proportions as they have in the blood. Therefore [illegible]

[illegible] led

1) The channels of the spectrometer checked with the standard preparations, must be stable before and after each separate part of the experiment

2) The volume record from the plethysmograph should give a smooth, readily measurable increase of volume during the periods *of venous congestion, exercise and histamine infusion.*

3) Because of the relative uncertainty of the assumptions on which the corrections for increases of regional blood volume during the filtration phases are based experiments where these corrections amounted to more than 10 per cent in volume were discarded.

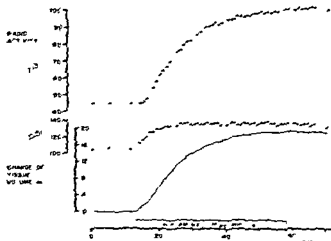


Fig 3 Exp 5 Change of activity emitted from I^{131} and Cr^{51} and change of tissue volume during a period of intra-arterial histamine infusion at a rate of 15 μg/min.

Results

Venous congestion The changes of tissue volume and of activity from I^{131} and Cr^{51} during a period of venous congestion in a representative experiment are shown in Fig 2. The volume curve shows a clearly biphasic course with an initial rapid increase followed by a phase of slower increase. The rapid phase corresponds to a steep rise of activity and is ascribed to an increase of the regional blood volume. The following slower phase is mainly caused by an accumulation of capillary filtrate. From the increase of Cr^{51} activity during this phase it was calculated that in this experiment 8 % of the increase of volume was caused by an increased regional blood volume while the remaining 92 % were ascribed to accumulation of capillary filtrate.

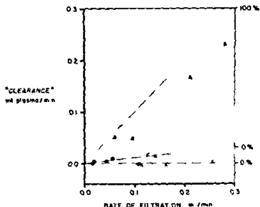
The content of albumin in the filtrate in this case amounted to 10 % ± 3 % of the albumin concentration of plasma. Table I gives the values for the albumin concentration of plasma and capillary filtrate in the 5 expts. The mean value for the albumin concentration of the capillary filtrate was 8.6 % of that in plasma or 0.18 g/100 ml. The error of determination was appreciable and resulted in two slightly negative values.

Exercise The right hand part of Fig 2 shows a representative experiment with induced muscular work producing a continuous high blood flow. In this case the increase of Cr^{51} activity indicated that the change of regional blood volume during the filtration phase was less than 2 % of the total volume change.

In Table I the results are listed. During exercise the albumin concentration averages 4.4 % of that in plasma or 0.14 g/100 ml of filtrate.

Histamine With histamine infused i.a. at a constant rate the volume increased rapidly together with the increase of blood flow but also continued to increase rapidly when blood flow had attained a high steady level. The activity from Cr^{51} rose initially but after a few minutes levelled off while the activity from I^{131} continued to increase at about the same rate as the volume. Fig 3 shows the results from infusion of 15 μg histamine per min in the experiment where the largest increase of volume occurred. Since the plots for the activity in the histamine experiment cannot be approximated to straight lines the concentration of albumin in the filtrate was calculated during several

Fig 4 Amount of plasma cleared from albumin per min plotted against the filtration rate Lines are given where the albumin concentration of the filtrate is 0 10 and 100 % of that in the plasma Filled circles denote values during venous congestion crosses during exercise and triangles during infusion of histamine (The filtration rate during histamine infusion in exp 5 was higher than 0 3 ml min, so the value is taken from the last part of that infusion where the filtration rate was lower but the protein concentration of the filtrate was the same as during the early part)



periods during each infusion and the values for ΔV, ΔR and ΔR were solved directly from the plots Values for A and B were taken from a period of venous congestion immediately preceding the infusion period In the experiment shown in Fig 3 the albumin concentration of the filtrate was calculated during three different periods and each time found to be about 80 % of that in plasma Table I gives the values obtained during 4 expts The mean value for the albumin concentration of capillary filtrate was 75 % of that in plasma or 2 0 g/100 ml of filtrate From the level of activity and the number of determinations the error of the change of activity of I^{131} was calculated to be below 3 %

Fig 4 shows the amount of plasma cleared from albumin per unit time plotted against the rate of capillary filtration in the three different types of experiments The values both from the periods of venous congestion and exercise lie close to the line of zero clearance, irrespective of the rate of filtration while all the clearance values are high during histamine infusion

Discussion

When this study was begun, the two methods earlier described were available for the determination of the protein concentration of the capillary filtrate None of them however, was entirely suitable for our purpose The method of Landis *et al* requires unphysiologically high congestive pressure and has given highly differing results in the hands of various investigators The method of Pappenheimer and Soto-Rivera is based on assumptions that are evaluated experimentally with difficulty

Theoretically, the advantage with the present method is that both the amount of capillary filtrate formed and the amount of albumin lost in a homogenous tissue muscle, are measured directly However since the albumin concentration of the filtrate during venous stasis and exercise is very low the changes of activity due to RISA in capillary filtrate are small compared to the activity from the intravascular RISA Therefore, the method only indicates the order of magnitude of albumin concentrations in the filtrate as long as this concentration is low Calculations of the standard deviation of the mean of 5—10 measurements at an activity of 40 000 cpm (the approximate level of I^{131} activity in the preparation) show that an accuracy better than ± 5 not

expected when the albumin concentration of the filtrate is below 10 % of that of plasma This is in agreement with the results in Table I

The present experiments show that the albumin concentration of the capillary filtrate formed during venous congestion or exercise is less than 10 % of that in plasma or about 0 16 g albumin/100 ml of filtrate This can be converted to total protein concentration of the filtrate if the protein content of the lymph is known In the steady state the relative proportions of proteins must be the same in the capillary filtrate the interstitial fluid and the lymph although the absolute concentrations may vary In an earlier study on the protein composition of lymph from the calf muscles of cat Jacobsson and Kjellmer (unpublished observations) found that 54 % of the lymph proteins were albumin versus 40 % in plasma With these values an albumin content of 0 16 g/100 ml filtrate corresponds to a total protein content in the filtrate of 0 30 g/100 ml This value is in good agreement with the lowest figures previously published *i e* those of Landis *et al* (1932) and Pappenheimer and Soto-Rivera (1948) Venous congestion and exercise did not give significantly different protein concentrations of the capillary filtrate This is in line with results obtained on the passage of dextran of varying molecular size through the capillary membrane during rest and exercise in skeletal muscle (Arturson and Kjellmer 1964) and corroborates the conclusion that the capillary permeability does not change during muscular work

The histamine experiments demonstrated that the described method allows separation between capillary filtrate formed with normal permeability of the capillary membrane and increased permeability Kjellmer and Odelram (1965) found that the capillary filtration coefficient (CFC) increased up to 6 times during infus on of large doses of histamine CFC as an indirect measure of the water conductivity thus increased less than the 12 fold increase of the permeability for proteins (*cf* Table I) Since the increase of CFC is due both to a larger capillary bed and an increased water permeability the difference in protein and water permeability is probably still more pronounced This is in keeping with other observations Arturson (1961) found a large increase of the permeability for large molecules in burned limbs while Arturson and Mellander (1964) only found a moderate increase of water permeability It is interesting to note that the simplest scheme for the function of the capillary wall — the theory of ultrafiltration through pores — predicts that an increased permeability that is an increased pore size will affect the passage of large molecules much more than small ones (*cf* Grotte 1956 Renkin and Pappenheimer 1957)

The present study also confirms that only a minor part of the volume change due to accumulation of capillary filtrate during venous congestion and exercise can be attributed to an increase of the regional blood volume from a gradual relaxation of the capacitance vessels The values from Fig 2 are representative for all the experiments The values of 8 resp 2 % of the filtration phase due to increase of reg onal blood volume are probably overestimations since an accumulation of activity occurs in badly perfused parts of the preparation mainly the skin which erroneously gives the impression of an increase of blood volume

References

ARTURSON, G, Pathophysiological aspects of the burn syndrome *Acta chir scand* 1961 Suppl 274 1—135

ARTURSON, G and I KJELLMER, Capillary permeability in skeletal muscle during rest and activity *Acta physiol scand* 1964 *62* 41—45

ARTURSON, G and S MELLANDER, Acute changes in capillary filtration and diffusion in experimental burn injury *Acta physiol scand* 1964 *62* 457—463

GROTTE, G, Passage of dextran molecules across the blood lymph barrier *Acta chir scand* [illegible]

KJELLMER, I, The effect of exercise on the vascular bed of skeletal muscle *Acta physiol scand* 1964 *62* 18—30

KJELLMER, I and H ODELRAM, The effect of some physiological vasodilators on the vascular [illegible]

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Fig 1 Main para aortic body of a 12-day-old thyroidectomized rat In this animal no residual thyroid was found Faintly chromaffin cell groups are seen in the body (Cr) Dichromate formol fixed frozen section at 90 μ × 100

Fig 2 Main para aortic body of a 12-day-old thyroidectomized rat A large bilateral residual thyroid was left Chromaffin cell groups (Cr) can be seen in the body Dichromate-formol Frozen section at 90 μ × 100

Fig 3 Main para aortic body of a 12-day-old sham-operated rat Some faintly chromaffin cell groups (Cr) can be seen. Dichromate formol Frozen section at 90 μ × 100

Fig 4 Main para aortic body of a 12-day old unoperated control Cr = chromaffin tissue A = aorta V = vena cava Dichromate formol Frozen section at 90 μ × 100



Fig 5 Main para aortic body of a 12-day-old hydrocortisone-treated rat Dichromate formol Frozen section at 90 μ × 100

Thyroidectomy was performed during the first day after birth under ether anesthesia partly under the preparation microscope

Sham operation was performed identically with thyroidectomy but the glands were left intact.

In the hydrocortisone group animals were daily injected subcutaneously with aqueous suspension of hydrocortisone (Hydroadreson Organon) from the first postnatal day Daily dose was 0 1 mg of hydrocortisone in 0 1 ml of NaCl

All animals were k [illegible] retroperitoneal tissue [illegible] parts of a pe[illegible] hrs Thereaf[illegible]



Fig 6 Thyroid gland of a normal 12-day-old rat van Gieson × 250

Fig 7 Thyroid gland of a 12-day-old hydrocortisone treated rat A reduction in the height of the follicular epithelium can be seen, van Gieson × 250

hrs The block was then washed in running water and immersed in 3 5 per cent neutral formaldehyde for about a week The formalin solution was changed several times until it stayed colourless Adrenal glands were removed from the block and weighed with a torsion balance The block containing both kidneys were then sectioned serially using freezing microtome at 40—90 μ and the sections were mounted on slides with glycerol jelly

A tissue block from the thyroid region containing the trachea and the laryngeal cartilages was removed and immersed in 10 per cent neutral formaldehyde for a week The thyroid gland was then dissected away and after weighing with a torsion balance dehydrated in alcohol series, carried through butanol to paraffin in a tissue processing machine embedded in paraffin wax and sectioned serially at 5 and 10 μ For staining Haematoxylin-eosin and van Gieson techniques were used (see §§ 609 and 708 in Romeis 1948)

The thyroid region of the operated animals was serially sectioned at 10 μ after paraffin embedding (see above) and examined for residual tissue The size of the residual thyroid tissue was roughly estimated by calculating the number of sections in which they were seen.

Results

Thyroidectomy

Only the 14 animals which survived without serious complications were used for further studies In these rats the existence of residual thyroid tissue was common, and only in 2 rats it was absent The others had small pieces of thyroid tissue left usually unilaterally but in 3 rats it was large and bilateral

The appearance of the operated animals did not differ from that of the sham-operated or unoperated controls of the same age and they gained weight normally

Disappearance of the extra adrenal chromaffin tissue took place in the same way as in normal controls Small groups of faintly chromaffin cells were seen in the main para aortic body but not elsewhere in the abdominal region No differences were noticed in the amount of chromaffin tissue in animals with different size of residual thyroid tissue (Fig 1 and 2) The amounts of chromaffin tissue were so small that quantitative measurements were not possible Difference in the disappearance of the extra adrenal chromaffin tissue between the operated and the control animals was so small if any, that it is hardly of any practical significance (Fig 3 and 4)

Hydrocortisone administration

In this group the animals gained in weight slowly and were small, loose skinned and shaggy at killing Haemorrhagic enteritis was a common feature

No signs of degeneration of the extra adrenal chromaffin tissue was noticed The bulk of the tissue was found in the main para aortic body (Fig 5), which gave a strong uniform chromaffin reaction Smaller strongly chromaffin bodies were found in the vicinity of the abdominal sympathetic ganglia and isolated chromaffin cell groups were also seen inside the pre and paravertebral ganglia

Weights of thyroids were less than normal in absolute terms, but in relative terms, [illegible]

between the experimental and normal animals

Discussion

The results obtained indicate that hydrocortisone in dosages used in the present study caused a depressive effect on the growing rat thyroid, although not a very radical one These findings are in keeping with the observations made by other investigators (see for ex Babikian 1964)

If now, as was supposed, hydrocortisone would affect extra adrenal chromaffin cells by inhibiting thyroid function, thyroidectomy should have prevented the postnatal degeneration of the chromaffin cells However, the differences observed in the amount of extra adrenal chromaffin tissue between the thyroidectomized and control animals were small, if any On the other hand, administration of cortisone sufficient to cause a drastic increase in the volume of the extra-adrenal chromaffin tissue, did not cause a complete inactivation of the thyroid, as judged histologically Therefore, it seems likely that the thyroid does not play, at least any essential role in the degeneration of the extra adrenal chromaffin cells

References

BABIKIAN L. G, Effect of hydrocortisone on thyroid structure in albino mice Nature (Lond) 1964 *201* 83

EPSTEIN D A CANTAROW G FRIEDLER and K E PASCHKIS Inhibition of thyroid function by cortisone and ACTH in hypophysectomized rats *Proc Soc exp Biol* (N Y) 1953 *82* 50–53

HOPSU V K, Effects of experimental alternations of the thyroid function on the adrenal medulla of the mouse *Acta endocr* (Kbh) 1960 *34* Suppl 48

LEMPINEN M, Extra adrenal chromaffin tissue of the rat and the effect of cortical hormones on it *Acta physiol scand* 1964 *62* Suppl 231

ROMEIS B, *Mikroskopische Technik R* Oldenbourg Munchen 1948

Acta physiol. scand. 1966. 66. 366—373

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Theory of the Heated Thermocouple Principle

By

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Abstract

Grängsjö, G., J. Sandblom, H. R. Ulfendahl and M. Wolgast. Theory of the heated thermocouple principle. Acta physiol. scand. 1966. 66. 366–373. — The heated thermocouple principle is widely used for blood flow measurements in tissues. A theoretical deduction of the relationship between the fluid flow and the response of the heated thermocouple is shown to be a hyperbola. Experimental values from model and animal experiments fit the theoretical curve quite well. It seems quite impossible to precalibrate the thermocouples due to the fact that they are surrounded by a nonperfused zone of injured tissue or noncirculating blood.

Gibbs (1933) introduced the heated thermocouple method for the measurement of regional blood flow and the method has since been used by many authors in different biological tissues with more or less success (Linzell 1953, Grayson 1952). During the last few years Hensel (Hensel and Ruef 1954, Hensel and Bender 1956) has been one of the most eager advocates of the method.

The difficulty of calibration is the greatest disadvantage of the method. Most authors have studied the relationship between the "heat conductance" and the blood flow and have found the relationship to be linear (Dosekun, Grayson and Mendel 1960, Hensel and Ruef 1954, Hensel and Bender 1956, Graf and Rosell 1958). Other authors have found the "conductance" not to be proportional to the flow (Linzell 1953, Graf, Golenhof and Hensel 1957, Graf and Stein 1958). In his pioneer model experiments Betz (1962) discussed the nonlinear relationship between the "heat conductance" and the blood flow. He pointed out the impossibility of precalibrating the thermocouples in artificial media, as did Graf and Stein (1958) also.

In the present investigation the shape of the calibration curve of the heated thermocouples is deduced theoretically and the result is compared with that of model experiments. A simplified form of the theoretically deduced equation is given here with reference to blood flow measurements in the renal cortex.

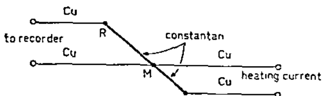


Fig 1 The figure shows the principles of the thermocouple probe Cu denotes copper wires. M is the heated point and R the reference point.

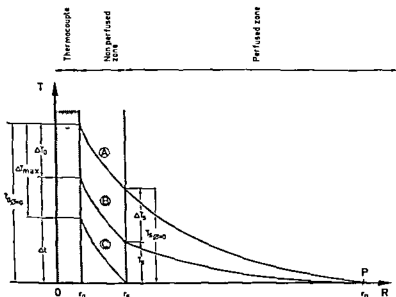


Fig 2 The temperature profiles surrounding the thermocouple T and R on the axis represent temperature and distance from the centre of the heated thermocouple respectively For explanation of the symbols see text Curve A shows the profile at zero perfusion flow ($v = 0$) and curve C at infinite flow ($v \to \infty$) Curve B represents an intermediate flow

The performance of the heated thermocouple

The heated thermocouple consisted of a constantan wire about 7 mm long 0 10 mm in diameter (Fig 1) To each end an insulated copper wire of the same thickness was soldered Two copper wires were soldered to a point on the constantan wire dividing it into a 3 mm part and a 4-mm part The short part was heated by an alternating current The temperature difference between the two thermojunctions at the ends of the long part of the constantan wire was recorded The probe used was similar to that described by Bill (1962)

Theory

Fig 2 shows the temperature field surrounding the heated thermocouple in a tissue The basal temperature of the tissue is set equal to zero. Immediately adjacent to the thermocouple appears a nonperfused zone probably caused by damage to the tissue in introducing the thermocouple

There is also a certain distance between the thermocouple and the nearest blood vessels depending of the distribution of the vessels, and this contributes to the size of the nonperfused zone.

Heat transport will only occur through this nonperfused zone by conduction

The following notation will be used

r_0 = radius of the thermocouple,

r_t = radius of the nonperfused zone,

r_p = distance from the centre of the thermocouple to the remote reference point, (point P in fig 2),

T_0 = Δt = temperature of the thermocouple,

T_t = temperature at the external surface of the nonperfused zone

ΔT = $\Delta T_0 = T_{0_{\phi=0}} - T_0$, where $T_{0_{\phi=0}}$ is the temperature of the thermocouple at zero flow,

ΔT_{max} = the value to which ΔT_0 tends when the flow is increased infinitely and

T_{max} = $T_{0_{\phi=0}}$ = the maximal temperature difference that can ever exist between the two thermojunctions with a certain heating current

Curve A in Fig 2 refers to the case when the perfusion velocity is made equal to zero and the transport of heat is pure heat conduction Curve B shows the temperature profile at a moderate perfusion velocity and curve C the temperature profile at a very large perfusion velocity

In the perfused medium the slope of the temperature profiles will in general be dependent on the flow but in the nonperfused zone where only conduction takes place the profiles will become parallel since the heat transfer is always the same if the heating current is kept constant Consequently the value of ΔT_0 for an arbitrarily chosen flow will be equal at the surface of the nonperfused zone ΔT_0 ΔT_t ΔT_0 and ΔT_t below are made equal to ΔT

As seen in Fig 2 ΔT_{max} (the decrease in temperature when the perfusion velocity flow tends to infinity will be the same as $T_{t_{\phi=0}}$ i e the surface temperature of the nonperfused zone at zero perfusion flow

With certain assumptions about the surrounding medium an expression relating the perfusion flow to the above mentioned temperatures can be derived as follows

In the first place the medium is assumed to have a homogeneous structure during perfusion Secondly the heat flow from the probe is assumed to be isotropic in all directions with the thermocouple at the centre of symmetry and only radial components of heat flow need therefore be considered Furthermore the reference point is regarded as being infinitely remote from the thermocouple

The heat transport $\dot{Q}$ cal sec within the medium is composed of two quantities, namely, the conduction Q_K and the convection $\dot{Q}_V$ which together give the total heat flow

$$\dot{Q} = \dot{Q}_K + \dot{Q}_V \tag{1}$$

The heat conduction $\dot{Q}_K$ is proportional to the gradient of temperature where the constant K defines the coefficient of heat conduction and is expressed in cal sec cm

$$\dot{Q}_K = -KS\text{grad } T \tag{2}$$

where S is a surface surrounding the thermocouple

The heat flow resulting from perfusion is obtained by multiplying the heat content of the medium with the radial component v of the perfusion velocity

$$Q_V = Svc T \varrho, \tag{3}$$

where ϱ denotes the density of the medium (g cm³ c its heat capacity cal g degree) and v its velocity (cm sec

Since both Q_K and Q_V are assumed to have the same direction the total heat can be written

$$Q = -KS\text{grad } T + SvcT\varrho \tag{4}$$

In a steady state the total heat flow across every surface which surrounds the probe is the same [illegible] passing [illegible] nce in [illegible] a long [illegible] centre [illegible] xternal [illegible] area of the nonperfused zone

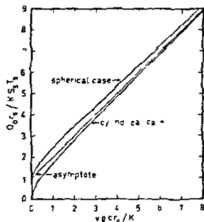


Fig 3 A graphical representation of equation (7), including also the spherical theoretical case and the asymptote to these two equations For explanation of symbols see text

Equation (4) can now be written

$$\frac{Q_o r_s}{r} = -KS_s \frac{dT}{dr} + S_s v \varrho c T \qquad (5)$$

To solve this equation, both sides are divided by K and S_s and multiplied by the integrating factor $e^{-\frac{v\varrho c r}{K}}$, which gives

$$\frac{Q_o r_s}{KS_s r} e^{-\frac{v\varrho c r}{K}} = \frac{d}{dr} T e^{\frac{v\varrho c r}{K}} \qquad (6)$$

This expression *can now be integrated between the following boundaries*

$$T = 0, \qquad T = T_s$$
$$r = r_p = \infty, \qquad r = r_s$$

Integrating equation (6) between these boundaries gives

$$\int_{r_s}^{\infty} -\frac{Q_o r_s}{KS_s r} e^{-\frac{v\varrho c r}{K}} dr = \int_{r_s}^{\infty} \frac{d}{dr} T e^{\frac{v\varrho c r}{K}} dr \quad -T_s e^{-\frac{v\varrho c r}{K}} \qquad (7)$$

The solution of this integral can only be obtained in terms of a series and must therefore be evaluated by numerical methods If the integral is regarded as a function of v it can be shown mathematically that the function has an asymptote for which equation (7) takes the form

$$\frac{Q_o r_s}{KS_s} = T_s \left(1 + \frac{v\varrho c r_s}{K}\right) \qquad (8)$$

By putting v = 0 on the right hand side of equation (8) we get

$$\frac{Q_o r_s}{KS_s} = T_{s_{\Phi=0}}$$

We also know that $T_{s_{\Phi=0}} = \Delta T_{max}$ and $T_s = \Delta T_{max} - \Delta T$

Substituting these relations and assuming v to be proportional to the total perfusion flow Φ, equation (8) may now be written

$$\Delta T_{max} \quad (\Delta T_{max} - \Delta T)(1 + k\Phi) \qquad (9)$$

where ΔT_{max}, according to equation (8) is $Q_o r_s / KS_s$ and where T and Φ are measurable quantities and k a constant $\left(\propto \frac{\varrho c r_s}{K}\right)$ The relationship between ΔT and Φ according to equation (9) is a hyperbola

By plotting the quantity $\Delta T_{max}/(\Delta T_{max} - \Delta T)$ as a function of Φ a straight line is obtained, according to equation (9) (Fig 3

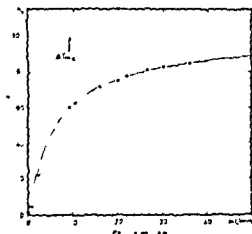


Fig 4 The hyperbolic relation between perfusion flow and ΔT is shown The open circles represent the experimental values and the curve the calculated hyperbola ΔT is expressed as a percentage of T_{max}

In the same figure the exact solution of equation (7) is represented together with the solution of equation 4 for the case of spherical symmetry i e when the thermistors are regarded as spheres It can be shown that the spherical case has the same asymptote expressed by equation (8) as the cylindrical case

For small perfusion flows the temperature profiles will extend further into the medium and the geometry is then expected to approach the spherical case

Since in both cases the functions converge rapidly towards the asymptote equation (8) may be considered approximately to describe the relationship between v and ΔT If $\dot{Q}$ and v are taken to denote vector quantities equation (4) is a very general equation and may together with the steady-state condition div $\dot{Q}$ = 0 be solved for any other set of conditions

The assumptions made in deriving equation (9) however are thought to be valid in most cases and experiments were made to test this theoretical model as below

Experiments

Methods

Model experiment A plastic tube 13 mm in diameter was filled with small glass beads varying between 80 and 100 μm diameter A Sigma pump forced water in a fairly steady flow through the system The flow rate was controlled by a measuring cylinder

The thermocouples placed in the centre of the tube were fed with the current from an AC power supply B B 138 The potential difference between the reference and the heated thermojunctions was recorded by a mirror galvanometer on a photokymograph All measurements were performed at room temperature

Animal experiment The heated thermocouples were used to study renal circulation in dogs (Grängsjö et al 196)

In anesthetized dogs the left renal artery was cannulated with a PVC tube and connected to the proximal end of one of the carotid arteries The connection was kept at 37 C by a

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The blood flow through the cortex was regarded as being proportional to the flow in the artery Kramer et al 1960

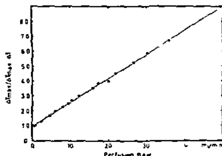


Fig 5 The linear relationship between $\Delta T_{max}/(\Delta T_{max} - \Delta T)$ and perfusion flow The open circles represent the experimental values

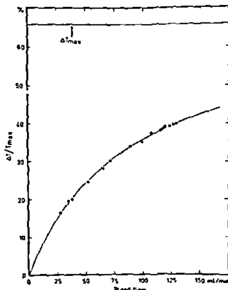


Fig 6 The hyperbolic relation between total renal blood flow and ΔT The open circles represent the experimental values and the curve the calculated hyperbola ΔT is expressed as a percentage of T_{max}

Results

Model experiments In Fig 4 ΔT from one experiment is plotted against the perfusion flow, Φ The hyperbola, according to equation (9), was calculated by the method of least squares In all model experiments T_{max} and ΔT_{max} coincided with each other, indicating a negligible nonperfused zone around the thermocouple The linear relationship between $\Delta T_{max}/(\Delta T_{max} - \Delta T)$ and the perfusion flow is seen in Fig 5

Animal experiments The results from the animal experiments were treated in the same way as those from model experiments Fig 6 shows the calibration curve from a renal experiment with the experimental points and the calculated curve. ΔT_{max} was always considerably lower than T_{max} and is drawn in the figure as the horizontal asymptote to the curve

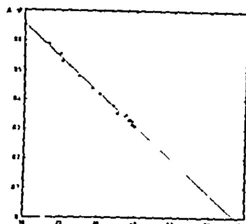


Fig. 7. The calibration line according to equation (10) with the thermocouple in the renal cortex. The values correspond to those in Fig. 6.

Equation (9) may be written

$$\Delta T \cdot \Phi = k \cdot \Delta T_{max} - k \cdot \Delta T, \qquad (10)$$

representing a straight line, if $\Delta T \cdot \Phi$ is plotted against ΔT.

When treated according to equation (10), the values in Fig. 6 give the result shown in Fig. 7.

The thermocouples have not been tested in other organs than the kidney.

Discussion

In order to deduce theoretically the relation between temperature changes, e.g. ΔT, and fluid flow, several assumptions were made. At least in the model experiments, the heat flow from the thermocouple was not actually the same in all directions but depended on the directed net flow given by the plastic tube. In spite of this, it seems to be valid to apply the experimental values to the derived formula (9). This seems also to be true of the renal cortex, where, however, the blood flow is probably more uniform in all directions.

As stated in the beginning, ΔT is the temperature change of the heated thermojunction when the perfusion flow is increased above zero. This ΔT is not analogous to the temperature change Δt used in most work with heated thermocouples. Δt is the temperature difference between the reference junction and the heated junction (Fig. 2).

Most authors have not given the relationship between Δt and the flow. They have instead related ΔK to the flow, where ΔK means the difference of "heat conductivity" between the actual and zero flow. $1/\Delta t$ gives a measure of ΔK. The hyperbola representing this relation converges to a straight line when the nonperfused zone is very small. This may be a reason why some authors have found a linear relation between ΔK and the flow, while others have not. The deviation from the straight line is pronounced when there is a thick haemorrhagic zone or when the tissue is badly vascularized.

A linear relationship may also be explained by (1) the use of only the steep, relatively straight part of hyperbolic standardization curve, (2) the opening of new vessels or (3) the dilatation of vessels (according to Bill 1962).

It seems quite clear that the shape of the calibration curve must vary from one case to another, depending on the heat transport by conduction (Q_K) or convection (Q_V) *It is impossible to precalibrate, as generally the Q_K and Q_V can never be predetermined with sufficient accuracy Thus the method requires calibration in situ*

References

Bill, A, Studies of the heated thermocouple principle for determinations of blood flow in tissues *Acta physiol scand* 1962 *55* 111—126

Dosekun F O, J Grayson and D Mendel, The measurement of metabolic and vascular responses in liver and muscle with observations on their response to insulin and glucose *J Physiol* (*Lond*) 1960 *150* 581—606

Gibbs, F A, A thermoelectric blood flow recorder in the form of a needle *Proc Soc exp Biol* (*N Y*) 1933 *31* 141—146

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Linzell, J L Internal calorimetry in the measurements of blood flow with heated thermocouples *J Physiol* (*Lond*) 1953 *121* 390—402

Shipley, R E and C Wilson, An improved recording rotameter *Proc Soc exp Biol* (*N Y*) 1951 *78* 724

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The Effects of a Gastric Inhibitory Substance on Gastric Secretion in the Pylorus Ligated Rat[1]

By

LARS S. SEMB[2]

Received 25 October 19[illegible]

Abstract

Semb L. S. *The effects of a gastric inhibitory substance on gastric secretion in the pylorus ligated rat.* Acta physiol. scand. 1966. 66. 374–382. — An extract was prepared from juice secreted by vagally denervated isolated pouches of the gastric antrum in the dog by dialyzing against water and lyophilization. This gastric inhibitory substance (GIS) was assayed in pylorus ligated rats. Three groups of rats served as controls. One group received no injections, while the two other were injected with saline 1 ml/100 g rat intravenously (i.v.) or intraperitoneally (i.p.). Two different types of gastric secretion were studied: (a) basal or spontaneous gastric output, and (b) gastric acid response to exogenous gastrin. GIS lowered significantly the basal or spontaneous output of acid but did not alter the basal output of pepsin significantly. When GIS was injected into animals stimulated by gastrin there was a highly significant lower output of both acid and pepsin.

Brunschwig and his associates have reported that alcoholic precipitates from human gastric juice or gastric mucosa inhibited gastric acid secretion when injected i.v. into dogs (Brunschwig *et al.* 1939, 1940, 1941a and b and 1942, Scott *et al.* 1943). The authors noted that the most pronounced inhibition was obtained from the gastric juice of patients with achlorhydria, and only to a lesser degree from extracts of normal gastric juice. The inhibition phenomenon has been confirmed by several investigators (Blackburn and Code 1948, Blackburn *et al.* 1950 and Smith *et al.* 1958). Gastric juice extracts from humans have been shown to inhibit gastric secretion in the rat (Mengus and Smith 1959, 1960, Katzka 1962, Nell and Barnard 1963).

A similar gastric inhibitory substance (GIS) was found to be present in the gastric juice of normal dogs (Livermore and Code 1952) and especially in the juice obtained

TABLE I Groups of animals

Group no	Treatment	Dosage	Route of adminis tration	Number of animals	Average weight (grams)
I	None	None	None	20	278 2
II	Saline	1 ml/100 g	i v	19	160 4
III	Saline	1 ml/100 g	i p	20	177 2
IV	GIS	2 5 mg/100 g	i v	18	129 7
V	GIS	2 5 mg/100 g	i p	20	209 5
VI	Gastrin	5 0 mg/100 g	i p	19	147 1
VII	Gastrin GIS	5 0 mg/100 g 2 5 mg/100 g	i p i p	20	179 1

from the antral portion of the stomach as compared to fundic juice (Hood et al 1953 Menguy et al 1963 a and b) The inhibitor has been demonstrated to be non-dialyzable (Smith et al 1958) Dialyzed and lyophilized extracts from canine gastric juice has been shown to inhibit basal (or spontaneous) gastric secretion in pylorus ligated rats (Menguy et al 1963) The effects of antral juice extracts on stimulated gastric secretion has not been studied in the rat In earlier studies the secretion of acid has been the main object of investigation, while only to a lesser degree has the gastric secretion of pepsin been studied Therefore this report is concerned with the effects of extracts from antral juice of the dog upon the basal and gastrin stimulated gastric secretion of acid and pepsin in the pylorus ligated rat

Methods

Inhibitory substance (GIS)

In this study GIS was obtained from 7 mongrel dogs prepared with vagally denervated isolated

mg ml

Gastrin

Gastrin was provided by T L Fletcher Ph D of the Research Laboratory of this Department

Animals

were

wide

Prior

All

rats tolerated this fast well and no deaths occurred The animals were divided into 7 experimental groups as indicated in Table I

Collection of gastric juice

[illegible] from the anesthesia within 2 to 3 min after withdrawal of the ether. After exactly 2 hrs, the animals were again ether anesthetized, the laparotomy wound opened and a clamp immediately placed at the oesophago-gastric junction to prevent reflux and spilling of gastric juice. The animals were then given an overdose of ether and sacrificed. By cutting the oesophagus, duodenum and the appropriate ligaments, the stomach was removed. The stomach contents were emptied into calibrated centrifuge glasses, cooled on ice and centrifuged in an "International Clinical Centrifuge" at approximately 2 000 rpm for 15 min. The supernatant was decanted and measured for volume.

Titration for acid

Acidity of the gastric juice was determined by titration against 0.01 N NaOH in a Fisher Automatic Titrimeter to pH 7.0 (Titratable Acid, TA).

Pepsin determinations

A modified An [illegible] was pipetted o [illegible] Biochemical C [illegible] adding 0.3 N [illegible] tometer at 280 [illegible]

Injections of the animals

I.p. injections were made after the laparotomy wound was closed by piercing the needle through the anterior abdominal wall above the symphysis pubis, taking care not to perforate viscera. I.v. injections were done before the closing of the laparotomy wound by inserting a No. 25 needle into the inferior vena cava. In all animals some bleeding occurred but gentle tamponade with a gauze pad soaked in saline controlled the bleeding rapidly. Some animals were discarded from the study because of too much bleeding. All animals were observed periodically for signs of shock or vomiting.

Rectal temperatures

These were measured by means of an electric thermometer to the nearest 0.1° C in 25 of the animals that received GIS, 9 received GIS and gastrin i.p., 5 received GIS i.p. and 11 received GIS i.v.

Weight of the animals

All animals were weighed immediately after the first anesthesia. Since there was a wide variation in the weight of the animals all experimental results obtained are expressed per 100 g rat (Madden *et al.* 1951).

Statistical analysis

Standard deviations from the mean (SD) and the significance of group differences, as judged by the "Students t test", were calculated according to Fisher (1958).

Results

All animals tolerated the laparotomy and the injections well. No deaths occurred, no signs of shock was observed and none of the animals had vomited in their cages.

The average results of volumes, titratable acid and pepsin are given in Table II, and the statistical comparison between averages are shown in Table III.

TABLE II Effect of gastric inhibitory substance (GIS) on volume, acid and pepsin in pylorus ligated rat stomachs

Treatment	Group no	Number of animals	Volume[1] ml/3 hrs	Total acid[1] Concentration µeq/l	Total acid[1] Output µeq/3 hrs	Pepsin[1] Concentration units/ml	Pepsin[1] Output units/3 hrs
Pyl lig	I	20	1 65 ± 0 15	56 0 ± 24 3	102 ± 77	175 ± 58	305 ± 136
Pyl lig + Saline i v	II	19	1 91 ± 0 89	63 4 ± 25 9	126 ± 81	141 ± 87	235 ± 154
Pyl lig + Saline i p	III	20	2 30 ± 0 27	80 0 ± 21 0	207 ± 109	225 ± 70	536 ± 131
Pyl lig + GIS i v	IV	18	1 50 ± 0 65	46 4 ± 19 1	75 ± 50	150 ± 61	209 ± 167
Pyl lig + GIS i p	V	20	1 47 ± 0 18	76 7 ± 24 3	124 ± 102	306 ± 103	480 ± 87
Pyl lig + Gastrin i p	VI	19	3 74 ± 0 22	94 7 ± 19 4	370 ± 31	416 ± 154	1 735 ± 283
Pyl lig + Gastrin i p + GIS i p	VII	20	2 77 ± 0 17	107 9 ± 20 1	301 ± 23	336 ± 119	951 ± 87

[1] All results are given per 100 g rat ± SD

TABLE III Statistical comparisons between group means

	II	III	V	VI	III	IV	VII	V	VI	VII	V	VII
	I	I	I	I	II	II	II	III	III	III	IV	VI
Volumes	NS	**	NS	***	NS	NS	***	**	***	NS	NS	***
$[\overline{TA}]$	NS	***	*	***	*	*	***	NS	*	***	NS	NS
$\bar{Q}$TA	NS	**	NS	***	*	*	***	*	***	*	NS	***
$[\overline{PU}]$	NS	*	***	***	***	NS	***	***	***	**	***	NS
$\bar{Q}$PU	NS	***	NS	***	***	NS	***	NS	***	***	**	**

NS = Statistically non significant ($p > 0.1$)
* = $p < 0.05$
** = $p < 0.01$
*** = $p < 0.001$

Volume of gastric juice

There was a significantly lower volume of gastric juice secreted by the animals that received no injections (I) compared to the group of animals that received saline i p (III) or gastrin i p (VI) In the groups of animals that received saline i v (II) or GIS i p (V) average volumes of gastric juice was not significantly different from those in the untreated group (I)

Injection of GIS i v (IV) resulted in a volume of gastric juice that was not different from the volumes secreted when saline was injected alone i v (II) In the animals where GIS was injected i p (V and VII) there was a significantly lower volume of gastric juice than in the respective control groups (III and VI)

Concentration of titratable acid ($[\widetilde{TA}]$)

Saline injected i v (II) gave no higher $[\widetilde{TA}]$ than was observed in the untreated group (I) but significantly lower values than in the group that received saline i p (III) Saline i p (III) gave a larger concentration than in the group of untreated animals (I) and gastrin i p (VI) increased the concentration even more

GIS injected i v (IV gave a significantly lower $[\widetilde{TA}]$ than in the control group (II) while GIS injected i p (V and VII) showed no effect on the $[\widetilde{TA}]$ as compared to controls (III and VI)

Output of titratable acid ($\widetilde{Q}_{TA}$

Saline injected i v (II gave a $\widetilde{Q}TA$ which was not different from the QTA in the animals that received no treatment (I) while it was significantly lower than in the group of animals that received saline i p (III) Injection of saline i p (III) resulted in a significantly higher acid output than in the group which received no injections (I) Gastrin administered i p VI and VII) gave the largest acid outputs as compared with appropriate control animals (III)

GIS injected i v IV) gave a lower $\widetilde{Q}TA$ than in the animals of the control group (II) The observed acid output average for group IV (GIS i v was not different from the average acid output observed in group V (GIS i p)

Administration of GIS i p in the pylorus ligated animals (V) gave a lower $\widetilde{Q}TA$ than in the control group III) but was not different from the untreated group (I) GIS given with gastrin i p VII resulted in a reduced $\widetilde{Q}TA$ as compared with controls (VI)

Concentration of pepsin $[\overline{PU}]$

I v injection of saline II resulted in a lower but not significantly different concentration of pepsin than in the untreated animals (I) Saline i p III gave increased $[\overline{PU}]$ as compared to untreated animals (I or saline i v treated animals (II) Gastrin i p (VI) gave a significantly greater $[\overline{PU}]$ than the control animals (III)

GIS i v resulted in a $[\overline{PU}]$ that was not different from the $[\overline{PU}]$ found in the animals that received saline i v (II) When GIS was given i p V) a larger pepsin concentration occurred than in the controls III) In the group given GIS i p together with gastrin i p (VII) the $[\overline{PU}]$ of gastric juice was not different from the average [PU] in the group that received gastrin i p alone (VI)

Output of pepsin ($\widetilde{Q}PU$

Saline injected i v II) gave no larger output of pepsin than observed in the untreated animals (I) When saline was injected i p (III) $\widetilde{Q}PU$ was larger than in untreated

animals (I) Gastrin injected i p (VI) resulted in greatly increased pepsin output as compared with controls (III)

In [illegible] GIS [illegible]
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animals (VII) resulted in a significantly lower $\bar{Q}$PU than in the group receiving gastrin i p alone (VI)

Rectal temperatures

Rectal temperatures measured before and 3 hrs after the injection of GIS in 25 animals are shown in table IV In only 7 of the animals was there any measureable increase in rectal temperature (0 1 to 0 3° C), and in 5 the temperatures were unchanged In the remaining animals the rectal temperatures 3 hrs after the injection of GIS were lower than before the injection (— 0 1 to — 0 4° C)

Discussion

Injection of saline i p apparently has a stimulatory effect on gastric secretion as indicated by the difference in gastric outputs in group III (saline i p) and group I (pylorus ligation alone) The mechanism behind this increased output in the group of animals treated with saline i p might be one of reversal of a state of dehydration in the rats caused by prolonged fasting This was first observed by Madden *et al* (1951) who found that gastric secretion in pylorus ligated rats diminished with increasing periods of fasting This occurred even if the animals were allowed water perorally in the fasting periods The authors also observed that such a decrease in gastric secretion could be reversed by injections of saline subcutaneously On the other hand this does not fully explain the effect of saline i p since in our series of animals receiving saline i v the gastric secretion did not increase significantly

The results in this study indicate that exogenous gastrin when injected i p into pylorus ligated rats stimulated gastric acid secretion This observation is in accord with earlier studies on the gastric acid response to s c injections of gastrin in the fistula rat (Adashek and Grossman 1962) The production of pepsin was also stimulated significantly This finding is in agreement with observations made in the dog (Dragstedt *et al* 1963)

Adashek and Grossman (1962) reported that in the fistula rat gastrin gave a larger maximal secretory response than histamine Other reports indicate that histamine has no effect on gastric secretion in the rat (Friedman 1943) while some authors report that histamine increases acidity but not volume of gastric secretion in the rat (Roe and Dyer 1939) Some investigators maintain that histamine is effective in the rat as a secretogogue but only when given in large doses (Komarov *et al* 1944 Kyle and Welbourn 1956 Valberg and Witts 1961) Ambrus *et al* (1953) found an inverse effect of large doses of histamine on rat gastric secretion with a decrease in volume and concentration of hydrochloric acid Because of these controversial reports and the reported observations on gastrin the conclusion might be drawn that gastrin is an effective secretogogue for studies of gastric secretion in the rat

The observation that an extract from canine antral juice injected i v into pylorus ligated rats inhibits the spontaneous gastric acid secretion Menguy *et al* 1963 a b) has been confirmed The present study has also demonstrated an inhibitory effect of i p

njection of such extracts on the spontaneous acid secretion in pylorus ligated rats The reduction in gastric secretion of acid in these groups of animals (IV and V) were of the same order of magnitude with an average of 39 % and 36 % reduction of acid output respectively when compared with controls (II and III) Furthermore, in our series of gastrin stimulated animals, the injection of GIS (VII) gave a reduction of gastric acid secretion when compared with the controls in which gastrin alone was injected (VI)

Previous reports from studies in rats (Menguy and Smith 1959) and dogs (Smith *et al* 1958) have shown that GIS derived from human gastric juice did not affect pepsin secretion These authors suggested that this indicated the specificity of GIS as an inhibitor of acid secretion In accordance with these reports there was no significant difference in the spontaneous output of pepsin from rats that received GIS i v or i p (IV and V) However in contrast to these findings, it was noted that GIS reduced the output of pepsin significantly in animals stimulated by exogenous gastrin (VII) Thus it is possible to assume that the inhibitory action of GIS is not solely confined to the acid secretory mechanism since it can also affect the pepsin secretion under certain conditions

Whether the inhibitions in these series of experiments are specific or unspecific cannot be determined conclusively from our data However, no deaths occurred after the injection of GIS no signs of anaphylactic shock were observed and none of the animals had vomited in their cages An important unspecific mechanism of gastric secretory inhibution was considered in this study namely that of hyperpyrexia In earlier reports on studies in both man and dog it was noted that hyperpyrexia will lead to inhibition of acid gastric secretion (Bandes *et al* 1948, Blickenstaff and Grossman 1950) Similar observations have been made in the pylorus ligated rat where pyrogens were shown to inhibit ulcer formation presumably by lowering the gastric acid secretion (McGinty *et al* 1949) A recent report shows that increased body temperatures induced by different methods affected gastric secretion in pylorus ligated rats (Brodie and Kundrats 1964) Further in some experiments on GIS in dogs a pyrogenic effect was observed following injections of the inhibitory extracts from gastric juice and it was shown that a probable correlation existed between the rise in body temperature and the degree of inhibition caused by the extracts (Livermore and Code 1952 Hood *et al* 1953)

According to Brodie and Kundrats (1964 an appreciable depression of gastric secretion was seen only in rats in which body temperatures were increased by 0 8—2 0° C In our experiments there was no significant temperature difference in the animals 3 hrs after the injection of GIS as compared with pre-injection levels In 7 of 25 rats the rectal temperatures increased by 0 1—0 3 C In consequence it does not seem probable that the inhibitions demonstrated in our studies were due to hyperpyrexia

Concerning the inhibition induced by GIS in the series of experiments with gastrin stimulated gastric secretion VII vs VI the effect of GIS could theoretically have been upon the spontaneous part of the gastric output on that part contributed to by exogenous gastrin stimulation or on both That at least some of the inhibition demonstrated is caused by a counteraction of the stimulatory effect of gastrin by GIS is indicated by the fact that in group VII there was a significant reduction in pepsin output while this could not be demonstrated in spontaneous secretion (IV and V)

The author is indepted to Dr Henry N ... man of the Department and Dr L M Nyhus ... ellent facilities and for giving valuable suggest... chief of chemistry research laboratories Dr T ... lying

...kewise I wish
to Drs Marcel
who all have

References

ADASHEK K and M I GROSSMAN Response of rats with gastric fistulas to injection of gastrin. Proc Soc exp Biol N Y 1963 112 629—631

AMBRUS J L C M AMBRUS and J W E. HARRISON Gastric acid secretion and ulcer formation of rats and guinea pigs Amer J Physiol 1953 173 95—99

ANDERSON W R, T L FLETCHER, R A MCALEXANDER, C. L. PITTS R L. COHEN and H N HARKINS, Preparation assay and preliminary characterization of bovine gastrin J Dairy Sci. 1961 44 2218—2226

ANSON M L., The estimation of pepsin trypsin papain and cathepsin with hemoglobin J gen Physiol 1938 22 79—89

B...

B...

BLACKBURN C. M C F CODE, C. P CHANCE and E E. GAMBILL, Confirmation of presence of a gastric secretory depressant in gastric juice of humans Proc Soc exp Biol (N Y) 1950 74 233—236

BLICKENSTAFF D and M I GROSSMAN A quantitative study of the reduction of gastric acid secretion associated with pyrexia Amer J Physiol 1950 160 567—571

BRODIE D A and S K KUNDRATS The effect of pyrexia on rat gastric secretion Gastroenterology 1964 47 171—178

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tion Arch Surg 1963 86 304—307

FISHER R A Statistical Methods for Research Workers 13th rev ed Oliver and Boyd Edinburgh 1958

FLETCHER T L W R ANDERSON C L PITTS R L COHEN and H N HARKINS A new preparation of gastrin Preliminary characterization Nature (Lond) 1961 190 448

FRIEDMAN M H F Histamine ineffective in rats as a gastric secretory stimulant Proc Soc exp Biol (N Y) 1943 54 42—44

HOOD R J Jr C F CODE and J H GRINDLAY Source of a possible gastric secretory inhibitor in canine gastric juice and effects of vagotomy on its production Amer J Physiol 1953 173 270—274

KATZKA, I., A gastric secretory inhibitor in normal and pernicious anemia Gastroenterology 1962 43 71—74

KOMAROV S A H SHAY M RAYPORT and S S FELS Some observations on gastric secretion in normal rats Gastroenterology 1944 3 406—410

K...

MADDEN R J H H RAMSBURG and J M HUNDLEY A critical study of the Shay rat Gastroenterology 1951 18 119—127

McGinty, D. A., M. L. Wilson and G. Rodney, The ulcer-inhibiting action of pyrogens. *Proc. Soc. exp. Biol. (N.Y.)* 1949 *70* 331

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inhibitory substance in gastric juice. *Fed. Proc.* 1963a *22* 343

Menguy, R. B., Y. F. Masters and W. A. Grybosky, Isolation of a gastric inhibitor substance in canine gastric juice. *Surg. Forum* 1963b *14* 339—340

Rheault, M. J., L. S. Semb, H. N. Harkins and L. M. Nyhus, Acidification of the gastric antrum and inhibition of gastric secretion. *Ann. Surg.* 1965 *161* 587—591

Scott, V. B., R. Moe and A. Brunschwig, Further studies on properties of the gastric secretory depressant in gastric juice. *Proc. Soc. exp. Biol. (N.Y.)* 1943 *52* 45—46

Shay, H., Komarov, S. A., S. S. Fels, D. Meranze, M. Gruenstein and H. Siplet, A simple method for the uniform production of gastric ulceration in the rat. *Gastroenterology* 1945 *5* 43—61

Smith, W. O., R. Hoke, J. Lanov, R. Caputto and S. W. Wolf, The nature of the inhibitory effect of normal human gastric juice on Heidenhain pouch dogs. *Gastroenterology* 1958 *34* 181—187

Valberg, L. S. and L. J. Witts. The histamine test meal in the rat. *GUT* 1961 *2* 32—36

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Spontaneous Impulse Activity in Immature Rabbit Brain

By

R M Bergström, Juhani Hyvärinen and Erkki Kurenniemi

In a study of the spontaneous impulse interval distribution in the retinal ganglion cells of a cat, Kuffler *et al* (1957) found that this is a gamma distribution Grossman and Viernstein (1961) established that the distribution of the cochlear nucleus impulses of the cat, in both the spontaneous and the excited state, is an exponential which indicates a simple random order of intervals In the same nucleus, Rodieck *et al* (1961) found not only the exponential, but also quasi gaussian and bimodal distributions Smith and Smith (1965) observed in the cat cerebral cortex a distribution resembling an exponential type with a long right hand tail

In the present study, the method of constructing impulse interval histograms has been applied to microelectrode recordings of spontaneous activity in the immature brain cells of the rabbit Extracellular recording with glass capillary microelectrodes was employed 29 new born animals, 0—14 days in age were tested The experiments were carried out under superficial urethane anesthesia, supplemented by local anesthetics The localization of the recording electrode was defined stereotaxically, and in most cases confirmed histologically by observation of the electrode track after formalin perfusion with the electrode in recording position In the data reduction system, the impulses triggered a time to-voltage converter monitored on an oscilloscope The same impulse also modulated the Z-axis, producing a bright unblanking spot at the end of each impulse interval (Fig 1) The height of the spot from the base line corresponds to the length of the preceding interval The material was thus photographed non stationary series were discarded and long series divided into stationary subseries These stationary series were then analysed with a pulse height analyser (Nuclear Data 160), and the histograms drawn with a digital plotter

An analysis was made of 26 stationary impulse series from the striatum 11 from the hippocampus, 4 from the hypothalamus and 3 from the cortex These series were recorded from 29 cells in all The resting membrane potentials were of the order of 5—20 mV corresponding to the values given by Mares (1964) for young rats In this extracellular recording many of the cells showed action potentials lasting up to 10 msec When such action potentials were encountered they were scarcely audible, but otherwise exhibited all the criteria of true extracellular biphasic action potentials with an amplitude of 1—2 mV By reason of the extracellular recording in the volume conductor, nothing precise can be said of the duration of the action potentials although they appeared to be a great deal longer than those in older animals with the same type of recording

None of the described well-defined types of histograms such as the exponential, gaussian, bimodal or gamma distribution were observed The histograms (Fig 2)

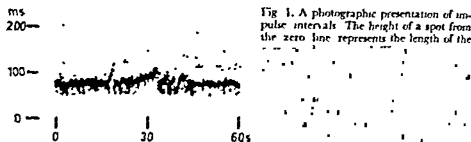


Fig 1. A photographic presentation of impulse intervals The height of a spot from the zero line represents the length of the

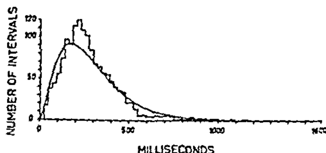


Fig 2 An impulse interval histogram of a cell in the globus pallidum of an 8-day-old rabbit Stationary sample of 1 448 impulse intervals lasting 4 min 45 sec mean impulse frequency 5.2 imp sec The solid line shows the best fitting theoretical gamma distribution for the same material

were always skewed to the right, although some of them were almost symmetrical They faintly resemble the gamma distribution, but bear a long right hand tail No obvious differences were apparent as regards the histograms from different nuclei or the studied age groups Fig 2 illustrates a typical histogram from a cell in the globus pallidum of an 8-day-old rabbit The theoretical gamma distribution for the same material has been indicated for comparison The chi-square test shows that the difference between the theoretical gamma distribution and the distribution arrived at empirically is significant at the 0 001 level The main differences are the height of the peak of the empirical histogram, its steep slope of descent, and the long right-hand tail

The results indicate that the spontaneous activity of immature brain cells is not simple random process alhtough it may contain random elements

This study was supported by the Finnish Medical Research Council

References

GROSSMAN R G and L J VIERNSTEIN Discharge patterns of neurons in cochlear nucleus *Science* 1961 *134* 99 101

KUFFLER, S W R FITZHUGH and H B BARLOW, Maintained activity in the cat's retina in light and darkness *J gen Physiol* 1957 *40* 683—702

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From the Physiological Institute University of Uppsala, Sweden

Convection, Diffusion, and Electric Current Through a Membrane

By

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Abstract

[illegible]

for the concentration of any ion in the membrane and solved using each of a number of simplifying assumptions Chloride conductance in the membrane was calculated with Eq 6, using the concentration of Cl in the membrane An observed value was obtained also from Eq 7 which related the driving forces on Cl to electric current The components of the membrane potential also were defined in the presence of water flow

Univalent ions kept at equal and fixed concentrations C_1, on Side 1 of a membrane, will attain equal concentrations on Side 2 C_2 if the system is permitted to attain equilibrium The application of a fixed hydrostatic pressure difference across the membrane, which produces water flow from Side 2 to Side 1, will reduce the *stationary state* concentration of each ion on Side 2 and lead also to *unequal concentration ratios* on Side 2 (ionic accumulation) if the mobilities of the ions differ in the membrane

The theory of ionic accumulation has been outlined by Hertz (1923) and Teorell (1951) and verified reviewed by Rapoport 1965) Addition of another fixed force, an electrical potential difference across the membrane, should change C_2 but not affect the accumulation ratio at the stationary state this prediction will be tested

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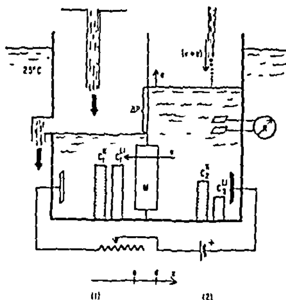


Fig 1 Experimental compartmental system, illustrated with $KCl + LiCl$ at stationary state Concentrations in Compartment 1 (C_1) were kept constant by a high rate of circulation of solution at $C_1 \cdot C_2$ variable ΔP, was maintained by overflow drainage in Compartment 1 and by dripping of distilled water from a capillary tube under a fixed pressure head, into Compartment 2 at $(v + e)$ ml sec Constant I was maintained by a high voltage D C. source and a high series resistance Positive direction from left to right along x axis Resistance between 2 Pt-black-Pt electrodes could be followed in Compartment 2 The figure shows relative accumulation of K Since water flow from the capillary was held constant, a change in membrane resistance to water flow would be associated only with a change in ΔP M = membrane e = estimated evaporation rate from Compartment 2, v = total water flow through membrane (in negative direction)

The ionic concentration profile in the membrane and the significance of membrane conductance will be considered in the presence of water flow The components of the total membrane potential and the signifiance of driving forces on the ions also will be analyzed

The metabolically dependent ability of a living cell to accumulate some ions and exclude others has not been clearly elucidated It is proposed that the convection-diffusion model will offer relevant information about the principles of accumulation and membrane conductance

Methods

Table of Notations

Units which include a may be converted to cm² membrane by appropriate division or multiplication by 3 5

- C = concentration where C_x = concentration/unit volume of membrane at x
- F = Faraday
- $\bar{V}^{H_2O}$ = partial molar volume of water (ml)
- RT = gas constant (joules/degree/mole) × absolute temperature
- φ_x = electrical potential (volts) at x in membrane, where $\varphi_1 = 0$ and φ_2 = transmembrane potential
- ξ_x = $\exp(zF\varphi_x/RT)$
- a = membrane area (cm²)
- d = membrane thickness (cm), $o < x < d$
- v = total bulk water flow through entire membrane in positive direction (ml/sec), where v/a = water flow/cm² membrane

i = designation for any specific ion
u = mobility of specific ion within membrane, under unit chemical potential gradient
η_x = exp $(-vx/RTu')$
D_0^{KCl} = diffusion coefficient of KCl in total membrane, when $v = 0$ (a cm²/sec)
f = RTu'/D_0^{KCl}
z = + 1 for univalent cation, —1 for univalent anion
R = integral membrane resistance (Eq 8)
E_{drv}^{Cl} = resultant driving force on Cl (volts) (see Eq 7)
R^{Cl} = Integral Cl resistance in membrane (Eq 6)
μ = electrochemical potential
V = $u C^- + u C$ (for anions)
U = $u C^+ + u' C'^+$ (for cations)
I = membrane current (a amps/cm²)
Φ_x = flux in positive direction through membrane (a moles/sec/cm²), where $\Phi_{\alpha\to\beta}$ = flux from α to β
κ = specific conductivity (mhos/cm)
ΔP = $P_2 - P_1$ (dyne/cm²), pressure difference across membrane

Experimental Procedures

Fig 1 illustrates the two compartment system A constant voltage power supply, fixed at 300 V and in series with a large variable resistance was used to obtain constant current conditions, I was measured by means of a sensitive galvanometer

Since a current shunt was produced when conductance was measured, κ_2 was obtained

and $d = 0\,013$ cm (by dry micrometer reading) Some characteristics have been described previously Surface area (a) for diffusion was 3 5 cm² and at 25° C water permeability was an average of 1 6 × 10 ⁴ ml/sec/cm water for the membrane

D_0^{KCl} was determined twice before and after each long term experiment by measuring the entry of KCl into Compartment 2 (C_1^{KCl} = constant, $C_1^{LiCl} = 0$) and by plotting $V\,d\ln[(C_1 - C_2)/C_1]$ against time the slope gave D_0^{KCl} (where V = volume of Compartment 2) The specific mobility, u, was obtained from D_0^{KCl} and from limiting ionic conductances, assuming a *nonselective uncharged* membrane When $f = RTu/D_0^{KCl}$ $f^K = 0\,982$ $f^{Li} = 0.517$ and $f^{Cl} = 1\,02$ (Rapoport 1965)

For an experiment with a chosen v the system initially was allowed to run for 12 hr with distilled water in both compartments, so as to obtain pressure-flow equilibrium, after which the water in Compartment 1 was replaced with rapidly circulating solution at C_1 A *fixed I* then was imposed until (1—3 days) constant κ_2 and C_2 (determined by consecutive sampling)

exception of exp 2 (see Fig 2)

Theory

The differential net flux equation for an ion in a wide pore membrane in which sieving may be neglected is

$$\Phi_i = -u_i C_i' [RT\, d\ln C_i/dx + z_i F\, d\psi/dx] + v C_i' \tag{1}$$

The integrated flux for a specific ion across a slice of membrane of thickness $\beta-\alpha$, where $\alpha < \beta$ is (cf. Rapoport 1965) when Φ is constant

$$\Phi_{\alpha\to\beta} = -RTu' \frac{(C_\beta' \xi_\beta \eta_\beta - C_\alpha' \xi_\alpha \eta_\alpha)}{\int_\alpha^\beta \xi \eta' dx} \tag{2}$$

At the stationary state if the net specific ionic flux across the membrane is zero, the specific ionic ratio C_2/C_1 is

$$C_2/C_1 = \xi_2^{-1} \exp(vd/RTu) \tag{3}$$

and the accumulation ratio A is defined (for K and Li in the experiments) (Teorell 1951, Rapoport 1965)

$$A = \frac{C_2^{K}/C_1^{K}}{C_2^{Li}/C_1^{Li}} = \exp\left\{\frac{vd}{RT}\left(\frac{1}{u^{K}} - \frac{1}{u^{Li}}\right)\right\} \tag{4}$$

In the presence of an applied potential and water flow at the stationary state $\Phi_{0\to x} = \Phi_{x\to d}$ $(0 < x < d)$ and C_x may be obtained as a function of ψ and v by use of Eq. 2

$$C_x = \frac{C_1\left[\xi_x^{-1}\eta_x^{-1}\int_x^d \xi\eta\, dx\right] + C_2\left[\xi_2\xi_x^{-1}\eta_2\eta_x^{-1}\int_0^x \xi\eta\, dx\right]}{\int_0^d \xi\eta\, dx} \tag{5}$$

Since ψ_x depends on the total ionic distribution in the membrane, it cannot be obtained explicitly in a two salt system. However, *in order to estimate* C_x^{Cl} in the experiments so as to calculate R^{Cl} (see below), the following approximations will be considered: (a) dC_x^{Cl}/dx is constant in the membrane $(0 < \psi_x < 0)$; (b) $\psi_x = 0$ and Eq. 5 is solved for C_x^{Cl}; (c) $\psi_x = \psi_2 x/d$ and Eq. 5 solved for C_x^{Cl} (constant field assumption); and (d) $v = 0$ and $\psi_x = \psi_2 x/d$ in Eq. 5.

The definition of the integral Cl resistance in the membrane is (Finkelstein and Mauro 1963)

$$R^{Cl} = \int_0^d \frac{dx}{F^2 u^{Cl} C_x^{Cl}} \tag{6}$$

Substitution of C_x^{Cl} derived under each of the above approximations in Eq. 6 will give an approximate *calculated* value of R^{Cl} and $1/R^{Cl}$ (Cl conductance).

In the experiments R^{Cl} was obtained also from an analysis of the electric current and the total driving forces on Cl at the stationary state. Under these conditions (see below), $I = -F\Phi^{Cl}$ and integration of Eq. 1 after the method used by Finkelstein and Mauro (1963) (cf. Teorell 1961) gives

$$I = -\frac{1}{R^{Cl}}\left\{\frac{RT}{F}\ln\frac{C_2^{Cl}}{C_1^{Cl}} - \psi_2 - \frac{vd}{Fu^{Cl}}\right\} \tag{7}$$

The three terms in the parenthesis constitute the net driving force on Cl, $E^{Cl}_{driving}$; the first term is the chloride potential E^{Cl}, and the second and third terms include the membrane and water potentials.

As will be shown, $E^{Cl}_{driving}$, as well as the net current I, is obtainable from experimental data so that an *observed* value of $1/R^{Cl}$ may be derived by Eq. 7 and compared to that calculated under the assumptions *a–d* from Eqs. 5 and 6.

TABLE I Relation of A_{observed} to $A_{\text{predicted}}$ (of the observed to the predicted accumulation ratio), when $I = 0$. $A_{\text{predicted}}$ was calculated from the right hand side of Eq. 4. $C_1 = (C_1^K + C_1^{Li})/2$ in mM. D_0^{KCl} was not determined in exp. 1. Mean of $A_{\text{pred}}/A_{\text{obs}}$ was 1.11. Values for exp. 2 and 3 have been given elsewhere (exp. 11 and 12 Rapoport 1965)

Exp no	$v \times 10^4$	C_1	$D_0^{KCl} \times 10^5$	A_{pred}	A_{obs}	$A_{\text{pred}}/A_{\text{obs}}$
1	−6.3	28	—	—	1.47	
2	−7.7	22	1.32	2.00	1.65	1.21
3	−8.5	23	1.56	1.91	1.53	1.25
4	−6.8	47	1.85	1.55	1.57	1.07
5	−10.0	45	2.28	1.58	1.54	1.03
6	0.0	45	2.28	1.0	—	

In the presence of water flow and a charged membrane φ_2 is given respectively by three terms — an IR drop, a diffusion potential and an electrokinetic potential — in the following equation for a univalent ionic system

$$\varphi_2 = -I\int_0^d dx/F^2(U+V) + \frac{RT}{F}\int_0^d d(V-U)/(V+U) + \frac{v}{F}\int_0^d\left[\left(\sum_{=1}^{n} C - \sum_{1}^{n} C\right)/U+V\right]dx \tag{8}$$

The integral in the first term is the integral membrane resistance and the equation need not be restricted to the steady state.

Results

Accumulation Ratio Eq. 4

Experiments in which the system had reached a stationary state were analyzed first in terms of the stationary state accumulation ratio of K to Li (Eq. 4) and the specific cationic ratio (Eq. 3) (see below). Previous experiments showed that Eq. 4 was correct to within 10–20% in the absence of electric current for the water flow and concentration ranges shown in Table I when ionic mobilities were estimated from D_0^{KCl} as described in Methods) (Rapoport 1965). For example Table I shows predicted and observed accumulation ratios for 6 experiments to whose numbers the paper will refer, when $I = 0$ and $\varphi_2 = 0$ to -5 mV (primarily a diffusion potential (see below)). As shown previously $A_{\text{observed}} < A_{\text{predicted}}$.

In the presence of electric current where $|\varphi_2| > 5$ mV Eq. 3 and 4 predict that the accumulation ratio but not the cationic ratio should remain independent of I or φ_2. Thus changing either I or φ_2 should permit alteration of the absolute values of C_2 without also affecting the respective cationic concentrations in Compartment 2.

To test Eq. 3 and 4 specific ionic ratios and the accumulation ratio were examined at a series of electric currents at a fixed stationary state v. Fig. 2 shows a typical experiment and indicates by observed x_2 values the evolution of a stationary state following a step

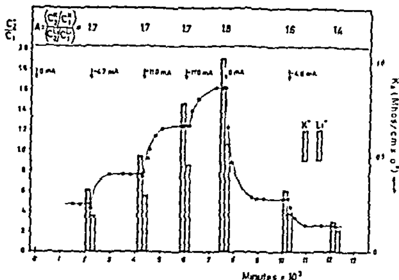


Fig 2 Stat state values for ionic ratios (C_2/C_1) of K and Li respectively and for the accumulation ratio at different electric currents Bars represent ionic ratios. Arrows indicate where I begins C_1^K $C_1^{Li} = 0.022$ M (Expt 2) When κ_t was constant a stationary state was established The ionic ratios increased with positive electric currents and decreased with negative I while $A_{observed}$ stayed approximately constant

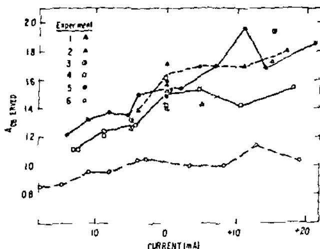


Fig 3 Relation of accumulation ratio to electric current Each slope of $A_{observed}$ on I was positive and those in Expts 4 5 and 6 significantly ($P < 0.05$) greater than zero

move-

and

Eq 4

strength of applied current (Fig 2 and 3) increasing with posit ve cu

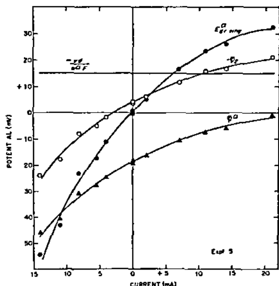


Fig 4 Relation between stat state membrane chloride and water potentials, and electric current Data from Expt 5 $E^{Cl}_{driving}$ was obtained by addition of the three curves φ_2 was derived from the cationic ratios, φ^{Cl} from the Cl ratios

does not predict this correlation possibly because it neglects residual membrane charge Although it has been shown that the relative diffusion coefficients of KCl, LiCl and glucose were not affected by the membrane (Rapoport 1965), the stat state Δ P increased with positive I in a manner expected from a negatively charged membrane in which electroosmosis takes place (Teorell 1959)

Specific Ionic Ratio Eq 3

It has been shown that Eq 3 is valid for the system at the stat state when $I = 0$ $\Delta P > 0$ and $v < 0$ Since, under these conditions, Cl , K and Li^+ are passive or "dependent" constituents of the system their respective net fluxes vanish (cf De Groot 1952, p 195), and Eq 3 will be valid for each of these ions At $I = 0$ from each of the observed specific ionic ratios and values of v and u a φ_2 was calculated by Eq 3, its mean was found and inserted back into Eq 3 and each of the u was adjusted for this insertion

The derived φ_2, at $I = 0$ was composed of a diffusion potential and an electrokinetic potential (Eq 8), and was not measured independently in experiments For the flows and concentrations shown in Table I, the electrokinetic contribution can be disregarded and φ_2 was calculated to within 10 % by neglecting water flow effects in either the Henderson or the Plank diffusion equations (Rapoport 1965)

Eq 3 was tested *for* $I \neq 0$ by noting that, under this condition, K and Li^+ were dependent constituents and Cl (as well as water) independent, since a stat state current maintained by Ag/AgCl electrodes is equivalent to a constant Cl current Values for φ_2 were *calculated* from each of the specific cationic ratios at each applied I by Eq 3 (using also v and u (adjusted)) *The φ_2 agreed with each other to within 10—20 % for Expts 1—6, thus confirming Eq 3 within these limits* (φ_2 could not be measured in the thin, wide pore membranes because of potential (IR) drops in the surrounding solu-

TABLE II Effect of electric current on Cl conductance ($1/R^{Cl}$) Exp 5 Observed conductance was obtained from $F^{Cl}_{driving}/I$ (Eq 7, Fig 4) Conductances in columns *a*–*d* were calculated by Eqs 5 and 6 under differing assumptions about water flow and electrical potential In columns *b* and *c* $c = -10^{-4}$ ml/sec Constant field means $\psi_x = \psi_2 x/d$ Constant concentration gradient in column *a*

		Chloride conductance (mhos)	*a*	*b*	*c*	*d*
I (mA)	C_2^{Cl}/C_1^{Cl}	Observed	$c = 0$, $\psi_x = 0$	$\psi_x = 0$	Constant field	Constant field, $c = 0$
−14.0	0.17	0.26	0.29	0.26	0.30	0.33
−11.0	0.20	0.25	0.31	0.29	0.32	0.34
−8.07	0.30	0.34	0.34	0.32	0.33	0.35
−5.38	0.34	0.31	0.38	0.36	0.39	0.40
−4.0	0.38	0.35	0.38	0.36	0.36	0.36
0	0.47	—	0.42	0.40	0.40	0.42
0	0.48		0.46	0.44	0.43	0.45
+2.0	0.52	0.41	0.47	0.45	0.45	0.46
+7.0	0.66	0.43	0.53	0.53	0.51	0.55
+10.9	0.76	0.46	0.52	0.52	0.51	[illegible]
+14.0	0.79	0.54	0.56	0.56	0.55	0.56
+21	0.96	0.61	0.60	0.60	0.60	0.60

tions) Insertion in Eq 3 of activity coefficients (coefficients cancel in Eq 4 taken from Kortum and Bockris (1951 p 680 did not change q_x values by more than 5% and did not change the limits of agreement

The q_x derived from $C_2^K C_1^K$ (using either concentrations or activities were less at positive I but greater at negative I than the q_x derived from $C_2^{Li} C_1^{Li}$ P 0.05 as would be expected from the variation of $I_{observed}$ with I

Membrane Conductance

In order to consider the relevance of Eq 5 to 8 to the system and to elucidate membrane properties under conditions of water flow and electric current the forces acting in the system and the meaning of membrane conductance will be analyzed

In Fig 4 the mean q_x calculated from the stationary specific Li and K ratios, is shown to depend non linearly on I Although such a q_x I curve may be analyzed in terms of an empirical differential conductance G $-dI/dq_x$ this conductance will lack a phenomenologically rigorous meaning in the experiments since it should be taken at constant c C_1 and C_2 (Michaeli and Kedem 1961 At the same time, concentration profiles should be undisturbed C_2 depended on I in the experiments and $I = -F\phi^{Cl}$, so that the total differential dI of Eq 7 contains several components when concentration profiles change (a similar differential obtains for Eq 8)

$$dI = d(1/R^{Cl}) \quad [E^{Cl}_{driving}] + (1/R^{Cl}) \quad d(\varphi^{Cl} - \varphi_2) \tag{9}$$

where $1/R^{Cl}$ and φ^{Cl} depend on C^{Cl}_x

Fig. 4 shows the components of the total driving force on Cl — obtained from observed stat state concentration ratios, v and u^{Cl} — and the resultant driving force E^{Cl}_{driv}, as related to I The water flow term is constant, while $-\varphi_2$ and φ^{Cl} vary in like manner with I The chord conductance, E^{Cl}_{driv}/I, equals an "observed" $1/R^{Cl}$

When C^{Cl}_x was obtained by means of each of the approximations (*a-d*), discussed in Theory, with Eq 5, and $1/R^{Cl}$ was calculated by Eq 6, the calculated $1/R^{Cl}$ differed by less than 10 % from the observed ones, column *a* +2 %, column *b* −3 %, column *c* + 7 % for all experiments Table II shows the results for Expt 5 whose driving force components are given in Fig 4

It is apparent that $1/R^{Cl}$ was set primarily by the variable boundary condition C^{Cl}_2, which was regulated by both v and φ_* (or I (Eq 3)) and apparently did not depend much on variations in concentration profiles for the v's and φ_2's chosen in the experiments For example $1/R^{Cl}$ calculated for a constant concentration gradient when $v = 0$ (Column *a*, Table II) was reduced only by about 0 02 mhos when $v = -1 \times 10^{-3}$ml/sec (Column *b*)

When observed $1/R^{Cl}$, obtained from Eq 7, was plotted against C^{Cl}_2, a linear relation obtained in the water flow experiments, linearity could not be demonstrated in expt 6 ($v = 0$)

Discussion

The formulations for the specific ionic ratio (Eq 3) and for the accumulation ratio (Eq 4) were studied under conditions of hydrostatic pressure differences across the membrane, producing water flow, and electrical potential differences producing current The experiments required that water flow and I be fixed parameters

Eq 3 and 4

since $A_{observed}$ 1

the fact that

were more negative at positive I and more positive at negative I, than the φ_2 derived from C^{Li}_2/C^{Li}_1

There are several possible causes for the deviations of Eqs 3 and 4 A negatively charged surface may interact specifically with univalent ions (Hober 1941, p 301) Neglect of charge in obtaining u may have produced errors in the estimation of f (Rapoport 1965) and thus in predicted concentration ratios In addition, the superimposition of positive electroosmotic flow due to positive currents (when J P increased) on the negative hydrostatic flow will change the flow profile of the latter in a pore (cf Davies and Rideal 196

stat state Ions adsorbed

convection term in Eq 1

of C_x

The assumption that u didn't depend on concentration is incorrect In addition errors may have been introduced by stirring variations or by heterogeneousness in pore size The latter condition implies that a macroscopic stationary state is a sum of microscopic systems which do not all have the same properties All the pore dimen-

sions were probably so large, however, with respect to ionic diameters that sieving or reflection was negligible

For the experimental conditions — $d = 0.013$ cm $C_i' = 0.015$ to 0.023 M, $I = +21$ to -18 mA — integral I/R^{Cl} obtained by Eq 6 with approximations *a*—*d* agreed with observed values obtained by Eq 7, to within an error that could have been due to errors in the estimation of q_i, q^{Cl}, u^{Cl} Thus the relatively thin membrane and high ionic concentrations made the testing of the approximations not entirely adequate A multicompartment membrane, in which q_x could be measured directly, would permit a better application of Eq 5 The constant field approximation (*c*) depends on a constant χ_x, which is best neared when $\chi_i = \chi_t$

Energy Dissipation

Under the [illegible]
open and ten[illegible]
Two generaliz[illegible]
$-I/F$ and bu[illegible]
separating dif[illegible]
$q^{Cl}\zeta^{Cl}$ and [illegible]
$I(q^{Cl} - q_t)$ joule/sec at the stationary state This energy is compensated by work done on the system which keeps the two independent forces at fixed levels and maintains the non zero fluxes

When the generalized force on a constituent is defined as $-\Delta_{\rho}\mu$ and if $-\Delta P$ is the force leading to bulk flow (mainly water flow in a wide pore membrane) the water potential in E_{driv}^{Cl} of Eq 7 is not part of the generalized force on Cl but arises by a cross (coupling) effect between q^{Cl} and $-\Delta P$ (cf Michaeli and Kedem 1961)

[illegible]

References

DAVIES J T and E K RIDEAL *Interfacial Phenomena* 2nd ed New York Academic Press 1963 pp 480

DE GROOT S R *Thermodynamics of Irreversible Processes* Amsterdam North Holland 1952 pp 242

FINKELSTEIN A and A MAURO Equivalent Circuits as related to ionic systems *Biophys J* 1963 *3* 215 237

HERTZ G Über Trennung von Gasgemischen durch Diffusion in einem stromenden Gas *Z phys* 1923 *19* 35—42

HÖBER R *Physical Chemistry of Cells and Tissues* Philadelphia Blakiston 1945 pp 676

KORTÜM G and J O M BOCKRIS *Textbook of Electrochemistry* Amsterdam Elsevier 1951 pp 882

MICHAELI I and O KEDEM Description of the transport of solvent and ions through membranes in terms of differential coefficients *Trans Faraday Soc* 1961 *57* 1185—1190

RAPOPORT S I Ionic accumulation by water flow through a membrane *Acta physiol scand* 1965 *64* 361—371

TEORELL T Zur quantitativen Behandlung der Membranpermeabilität *Z Elektrochem* 1951 *55* 460—469

TEORELL T Electrokinetic membrane processes in relation to excitable tissues II *J gen Physiol* 1959 *42* 847 863

TEORELL, T The ion flux across membranes during electro-diffusion and convection *Acta physiol scand* 1964 *62* 293—294

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Some Bioelectrical Properties of Amoeba proteus

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Abstract

Josefsson, J O *Some bioelectrical properties of Amoeba proteus* Acta physiol scand 1966 *66* 395—405 — The membrane potential resistance and capacitance of *Amoeba proteus* was measured with intracellular microelectrodes Specific membrane resistance was 3 700 ohmcm² [illegible]

brane potential and decrease of membrane resistance simultaneous with the spontaneous emptying of the contractile vacuole was recorded The hyperpolarizing component of this potential was evaluated in the light of a positive potential recorded during the impalement of the vacuolar membrane and the subsequent emptying of the vacuole All agents with ability to induce pinocytosis which were tested caused a transient "burst" of depolarizing potentials before a steady depolarized state was established Anionic compounds did not depolarize the membrane. It is suggested that the permeability of the cell membrane is largely governed by negatively charged groups of the mucopolysaccharide layer of the membrane

For several decades potential differences have been recorded across the cell membrane or walls of large protozoa However, only recently techniques have become sensitive enough to permit reliable measurements Glass capillary microelectrodes have been used by Bingley and Thompson (1962) in *Amoeba proteus* and by Riddle (1962) in *Pelo-* [illegible]

ed a potential difference between different parts of the cell A pseudopod was positive with respect to the cell body but negative with respect to the external solution

Large spontaneous variations of the membrane potential have been demonstrated in amoeba (Tasaki and Kamiya 1964) In the ciliate *Paramecium caudatum*, Yamaguchi (1960) noted a regular variation of the potential near the contractile vacuole, synchronous with emptying The present investigation of *Amoeba proteus* reports the effects of

external ions on the membrane potential studied with capillary microelectrodes. Estimates of the membrane resistance and capacitance were made and there were rhythmical potential changes recorded during the activity of the contractile vacuole.

Material and methods

Amoeba proteus Bristol was obtained from the Carlsberg Laboratory, Copenhagen. Stock cultures maintained at 20° C were fed on Tetrahymena (Prescott 1956, Chapman-Andresen 1958). The culture medium (Pringsheim) had the following composition (mM): Na^+ 0.22, K^+ 0.35, Ca^{++} 0.85, Mg^{++} 0.08, Fe^{++} 0.007, $HPO_4^{=}$ and $H_2PO_4^-$ 0.11, Cl^- 0.35, NO_3^- 1.70, $SO_4^{=}$ 0.09. Divalent ions and NO_3^- were omitted during observations of the effects of the altered external ionic composition. The amoebae were starved for two days prior to study to free them from ingested Tetrahymena.

Experiments were performed in a 5 ml perspex chamber in which the amoebae were allowed to attach themselves to the bottom. For recording over long periods the amoeba was detached from the glass by small movements of the impaling microelectrode and elevated into the bathing solution. The medium could be changed without disturbing the amoebae. In each of the experiments new amoebae were used.

For intracellular recording and application of current pulses conventional glass capillary microelectrodes were used. The electrodes were filled with 0.3 M KCl instead of the usual 3 M KCl solution (Ling and Gerard 1949) after it was found that the lower concentration of potassium chloride increased the stability of the recorded potentials and decreased the size of the electrode tip potential. The tip potential of recording electrodes was less than 10 mV and their electrical resistance measured in 0.3 M KCl was 40—80 MΩ. Conventional Ag-AgCl electrodes in Pringsheim-agar were used as indifferent electrodes. The amoebae were impaled from above under visual observation with a binocular microscope at 100 × magnification. [illegible] and a grid current of [illegible] rode [illegible] rode [illegible] peri-ment was discarded. The recorded intracellular potentials were displayed on a dual beam oscilloscope (Tectronix model 502) and photographed. An inkwriting oscillograph was also used for recording of slow potential changes.

"Input" resistance of the amoeba was determined as described by Fatt and Katz (1951). The potential change across the membrane was measured with the recording electrode when square pulse currents of 10—60 msec duration were delivered from a Grass Stimulator of Model S4 through a second microelectrode inserted into the cell. At the output of the stimulator a 200 MΩ resistor was in series with the current electrode in order to eliminate changes in current flow. An indifferent electrode was grounded through a resistor and the potential drop across this was monitored on the oscilloscope. With these arrangements hyperpolarizing pulses 1—3 per second were used for continuous recording of membrane resistance during periods of several minutes. No changes in resting potential due to electrophoretically altered ionic composition of the cytoplasm could be noticed in these experiments.

Results

Membrane potential

The membrane potential was recorded in Pringsheim solution at 18—20° C. It varied somewhat in cells from different culture dishes but was always negative with respect to the surrounding medium. Upon insertion a potential of about — 30mV was recorded. In most cases puncture was followed by a slowly increasing negativity which reached [illegible] the electrode had to be moved further [illegible] values given for membrane [illegible] S.E. of mean in 25 amoebae being 65 ± 1.8 mV.

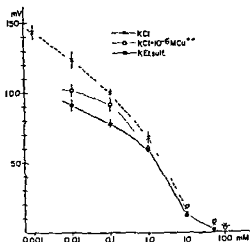


Fig 1 Relation between membrane potential and external K+ concentration when chloride or ethanesulphonate are used as anions In one series of experiments $CuSO_4$ was added to give 1 μM Cu^{++} ions in the external potassium chloride solution Bars denote standard error of the means

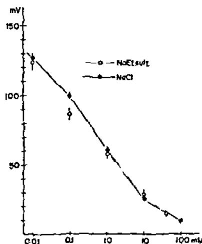


Fig 2 Relation between membrane potential and external Na+ concentration when chloride or ethanesulphonate are used as anions Bars denote standard error of the means

No feature of the gross morphology of the amoeba could be correlated with the value of the membrane potential Bingley and Thompson (1962) recorded a 30 mV potential difference between the inside of a pseudopod (more positive) and the uroid In the present experiments the membrane potential of pseudopod was also found to be 10 to 30 mV positive to the center of the cell However electrotonic potentials recorded in the pseudopod usually lacked a proper capacitative component indicating that a true intracellular insertion of the tip of the electrode may not have been achieved at that site Spontaneous streaming of the cytoplasm towards or away from the recording electrode did not affect the membrane potential

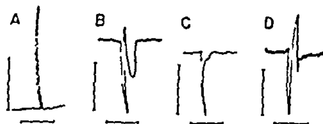


Fig 3 Vacuolar emptying potentials A High negative membrane resting potential specimen in distilled water B—D Specimens in Pringsheim solution RP A 130 B 40 C 30 D ?? mV ins de negative Downward deflection is negative in all records Voltage calibration A 50 mV, B—D 30 mV Time A—C 60 sec D 15 sec

Effects of changes in the ionic composition of the external medium

The membrane potential was found to vary with the logarithm of the external potassium concentration irrespective of whether the anion was chloride or ethanesulphonate (Fig 1) In the range between 0 1 and 10 mM potassium chloride the slope of the potential change was approximately 40 mV for each log unit change in the external potassium concentration As shown in Fig 1 the addition of copper sulphate in a concentration of 10^{-4} M somewhat increased the linearity of the curve This observation is possibly explained by a decrease in the chloride conductance of the cell membrane similar to that reported in the frog s skin after exposure to copper ions (Koefoed Johnsen and Ussing 1958

In bathing solutions containing sodium chloride or sodium ethanesulphonate a linear relationship was found between membrane potential and the logarithm of the external cation concentration As shown in Fig 2 the depolarization was approximately 35 mV for each tenfold increase in sodium concentration

These changes in membrane potential were not seen by simple sucrose induced increase in osmolarity of the external fluid

Electrical membrane resistance and capacitance

The input resistance of the amoeba was determined in Pringsheim solution by passing a square wave current pulse through the cell membrane while recording the current and the membrane potential change With correction for resistance of the external fluid the input resistance in 40 amoebae was 2 9 ± 0 4 MΩ mean ± S E of the mean) The figure can be transformed to the specific membrane resistance Rm ohmcm²) if the surface area of the amoeba is known Unfortunately this cell has no geometrically defined shape and hence its surface area can only be estimated The usual procedure was to disturb the protozoon mechanically until it converted itself into a spherical shape whose diameter could be measured The average surface area thus obtained was 1 26 × 10^{-3} cm² and the average specific membrane resistance 3 700 ohmcm²

With polarizing currents up to 0 01 μA a linear relationship was obtained between the amplitude of the electronic potential and the current indicating that the membrane behaved as a passive resistive component

The average electrical time constant of the membrane (Tm) as measured from the rising and falling phases of the electrotonic potential was found to be 6 7 msec Membrane capacitance (Cm) was obtained from the relation Tm = Rm × Cm and averaged 1 9 μF/cm² in 40 amoebae

TABLE I Relation between the predominant shape of "emptying" potential (EP) and the resting potential (RP) of the amoeba

Shape of EP	RP mV (mean±S E M)
Hyperpolarizing	49±2 2 (29)[1]
Biphasic	67±5 2 (17)
Depolarizing	78±4 9 (26)

[1] The figures in brackets indicate the number of amoebae

Potential changes simultaneous with the emptying of the contractile vacuole

Spontaneous rhythmic potentials were recorded in most amoebae The shape of these potentials in Pringsheim solution varied from monophasic depolarizing or hyperpolarizing potentials to polyphasic ones (Fig 3) Each amoeba had a single characteristic and recurrent potential which had a period of about 4—5 min

A vacuolar "emptying" potential of a depolarizing type was more common at high resting membrane potentials than were biphasic or hyperpolarizing emptying potentials (Table I)

The form and amplitude of the vacuolar emptying potential remained unchanged if the tip of the electrode was not displaced (Fig 4) The potential preceeded the contraction of the vacuole by less than one second The sequence of the electrical and morphological events lasted about ten and the total time varied in proportion to the size of the vacuole being emptied A short hyperpolarizing phase usually preceded a longlasting depolarization Identical emptying potentials could be recorded with electrodes in different parts of the cell (Fig 5) Potentials synchronous with the systoles of the contractile vacuole were recorded at every insertion in living amoeba The time for diastole varied from three minutes in distilled water, four minutes in Pringsheim to more than 10 min in 75 mM sucrose No specific monovalent ion had a predominant effect on the frequency of systoles which could not be explained by the osmotic effect exerted

G-strophanthin in a concentration of 10^{-4} w v, known to block active cation transports in some metazoan cells (Schatzmann 1953) had no effect even when applied for a period of 1 hr The resting membrane potential the configuration, amplitude and frequency of the vacuolar emptying potential were unchanged

During the depolarizing phase of the emptying potential the "input" resistance measured by the transient response technique declined to a steady value sometimes less than a fifth of normal (Fig 6 a) The filling of the vacuole was not accompanied by significant changes in "input" resistance

The potential recorded inside the contractile vacuole

In order to find an explanation for the biphasic potential recorded during emptying, attempts were made to record the potential between the inside of the vacuole and the external medium The procedure was to stop the vacuole from emptying with proteose peptone (Chapman Andresen 1962) and then to transfer the amoeba to Pringsheim solution as soon as the vacuole was large enough to permit microelectrode insertion.

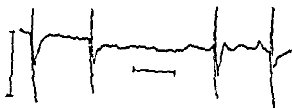


Fig. 1 Continuous intracellular recording of the membrane potential. Spontaneous recurrent vacuolar emptying potentials probably indicate emptying of two vacuoles twice. Voltage calibration 30 mV. Time 60 sec

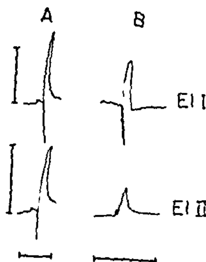


Fig. 5 A. Emptying potential recorded in one amoeba with intracellular electrodes (I and II) about 50 microns apart. Resting potential at both [illegible] Time 3[illegible] for the [illegible] outer [illegible] The resting membrane potential is 30 mV at this electrode and 60 mV at electrode I

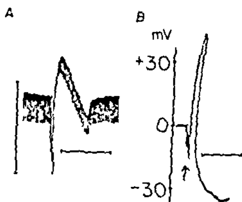


Fig. 6 a. Input resistance of an amoeba during emptying of a vacuole. Constant continuously monitored recurrent hyperpolarizing current pulses (not shown in the record) were unchanged during the experiment. Downward deflection from the baseline at each current pulse is proportional to the input resistance. A marked reduction in resistance occurs during the prolonged depolarizing phase of the emptying potential. Voltage calibration 50 mV. Time 30 sec. b. Potential observed during insertion of a microelectrode (arrow) into a contractile vacuole enlarged by immersion of the amoeba in a 0.1 per cent solution of proteose peptone for 2 min. Time 60 sec

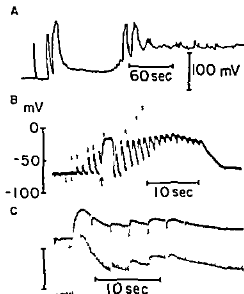


Fig 7 a Continuous intracellular recording of membrane potential from an amoeba in alcian blue solution (10^{-4} w/v) b Membrane potential changes induced by repetitive microapplication of alcian blue to the external surface of an amoeba 30 msec pulses 10^{-7} A each were repeated at 1 sec intervals. Release of dye between pulses was prevented by a small braking current. Stimulus artefacts are seen at each pulse. Membrane response begins at the 4th stimulus (At the

to the second and subsequent applications Iontophoretic current 10^{-7} A pulse duration 80 msec Voltage calibration 50 mV Stimulating conditions otherwise as in fig 7 b recording conditions as in fig 6 a

The insertion of the electrode always caused the vacuole to empty This was accompanied by a mainly monophasic potential (Fig 6 b) sometimes amounting to + 50 mV The time course of the potential was similar to that recorded when the intracellular electrode was outside the vacuole After the positive potential had declined the membrane potential slowly returned to its original resting value Similar results were obtained when chlorpromazine 10^{-4} w/v was used to delay systole Attempts to record from vacuoles without pretreatment were not successful

Effects of pinocytosis inducers

Rustad (1959) and Chapman Andresen (1962) have described specific substances which can induce pinocytosis Some of these were added to the external medium while recording the membrane potential of the amoeba Concentrations of inducers optimal for pinocytosis brought about almost complete depolarization of the cell At times a slightly positive potential was observed Before this final steady state was reached shortlasting depolarizations could be recorded (Fig 7 a) No morphological study of pinocytosis was undertaken

External iontophoretic microapplication of cationic inducers of pinocytosis gave prompt brief depolarizations resembling those seen when the vacuole emptied through the cell membrane (Fig 7 b) The response was exhausted by repetitive application unless intervals of 10 min or more were allowed During depolarization the membrane resistance was reduced to one half (Fig 7 c)

Rustad (1959) and Chapman Andresen 1962 have shown that certain cations induce pinocytosis In the present study only cations were effective in producing depolarization upon iontophoretic application Negatively charged compounds such as heparin, which do not induce pinocytosis (Chapman Andresen 1962) lacked effect

TABLE II Effect of compounds applied iontophoretically to the external surface of the amoeba The cells were immersed in distilled water or in Pringsheim solution of different pH

Depolarizing	No effect
Acetylcholine	γ Aminobutyric acid
Glutaminic acid (pH 7 5)	*Histidine*
Aspartic acid (pH 7 5)	Heparin
Alcian blue (Gurr)	Adenosinetriphosphate
Protamine	Glutaminic acid (pH 5)
Compound 48/80	*Aspartic acid (pH 5)*
Ribonuclease (Armour)	
Na^{+} K^{+}	

on membrane potential as did all anions of the tested salt solutions The currents used in these experiments were between 10^{-8} and 10^{-7} A The effects of the tested substances and ions are summarized in Table II

Histidine in free amino acid form does not induce pinocytosis (Chapman Andresen 1961) In the present study it did not cause membrane depolarization when applied to the cell surface However, in a concentration of 10^{-8} w/v, it produced shortlasting spontaneous depolarizations unrelated to observable morphological changes in the amoeba These potentials were always monophasic and of shorter duration than those accompanying vacuolar emptying

Discussion

Various values of the membrane potential have been observed in *Amoeba proteus* The most recent is given by Bingley and Thompson (1962) who in Chalkley's medium recorded a mean membrane potential of 72 mV This value is slightly higher than that obtained in the present investigation in Pringsheim solution The difference may be the result of variations in the ionic composition of the two external media

Difficulty in obtaining a proper intracellular insertion of the tip of the microelectrode was illustrated by the recording of a two step membrane potential change The first potential was presumably due to the pressing of the tip of the electrode against an invaginated cell membrane while the second and higher potential level is reached when the electrode actually penetrated the membrane Another explanation of this phenomenon is compartmentalization of the cell with electrical potential differences between the compartments The potential of the ectoplasm might be lower than that of the endoplasm thereby producing the stepwise increase in the negative potential when the tip of the electrode was inserted into the interior of the amoeba In a pseudopod only the first step of the membrane potential was recorded and it is therefore uncertain whether the value represents a true intracellular potential Under these circumstances it is not possible to give a meaningful and physiological interpretation of the potential difference between parts of the cell such as the pseudopod and the uroid

From the values of membrane potential when the ionic composition of the external solution is varied it may be concluded that the membrane is fairly permeable to mono-

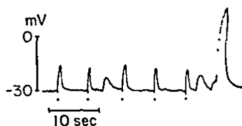


Fig 8 Intracellular recording of the membrane potential from a protozoa, *Vorticellae sp* The record shows spontaneous vacuolar emptying potentials indicated by dots and potential changes occurring simultaneously with the myoneme contraction The large potential resulting from complete coiling of the myoneme is shown to the right in the record

valent cations In contrast to many metazoan cells, it shows no preferred permeability to potassium The failure of the membrane to behave as a potassium electrode has also been recognized by Bingley and Thompson (1962) and Bingley (1964) and in *Pelomyxa carolinensis* by Riddle (1962) The observation that substitution of chloride with ethanesulphonate, a much larger anion, had no effect on the membrane potential suggested either that the permeability of the membrane to chloride is low or that the membrane is equally permeable to ethanesulphonate

The ionic composition of the amoeba is unfortunately, not known In *Pelomyxa carolinensis*, the potassium concentration during culture in Pringsheim has been estimated to be about 35 mM/l (Riddle 1962, Chapman-Andresen and Dick 1962), and the concentration seems to be similar in *Amoeba proteus* (Johansson and Josefsson, unpublished) Presumably the concentration gradient must be maintained by active inward ion transport *During culture conditions where sodium concentration in the medium* is 0 22 mM there appears to be no need for an energy dependent sodium transport system, such as that in metazoa This hypothesis is supported by the observation that g-strophanthin was without effect on the membrane potential, and by findings in *Acanthamoeba sp* (Klein 1964) where sodium is reported to be freely diffusable and the active inward transport of potassium is not influenced by ouabain

Nothing is known about the composition of the fluid in the vacoule, which seems to be *bounded by a typical unit membrane* (Greider *et al* 1958) Schmidt-Nielsen and Schrauger (1963) have shown that the osmolarity of the vacuolar fluid is 32 mosm compared to 100 mosm of the protoplasm

The recording of a potential change during vacuolar emptying is not unique to the amoeba Other protozoa with vacuoles exhibit similar changes in membrane potential during contraction Yamaguchi (1960) found a hyperpolarizing potential during vacuolar emptying in *Paramecium caudatum* Recently we have found that species of *Stylonychia* and *Vorticella* exhibit changes in membrane potential during contraction of the vacuole In the latter potential changes could be recorded during the contraction of the myoneme in addition to the vacuolar emptying potentials (Fig 8)

There are at present few experimental data upon which to base a hypothetical explanation for the ionic shifts responsible for the vacuolar emptying potential The ionic composition of the vacuolar fluid presumably differs from that of the protoplasm and therefore an electrochemical gradient would exist across the vacuolar membrane This gradient might influence the total membrane potential at the moment of vacuolar emptying when the vacuolar membrane transiently becomes a part of the cell surface A tentative explanation for the shape of the emptying potential may be that the vacuolar content had a positive charge relative to the protoplasm, possibly as the result of active transport of cations into or anions out of the vacuole At the moment of emptying the

electromotive force of the vacuolar membrane would increase the membrane potential in proportion to the electromotive forces and to their active membrane areas. The depolarization in the last phase of contraction may represent a disruption of the cell membrane leaving only the vacuolar unit membrane as a diffusion barrier to the exterior at the site at which the vacuole empties into the external medium.

If the emptying potential is produced in this way the monophasic depolarization would predominate at high resting potentials. Biphasic potentials, first phase negative, would be recorded until the resting potential is low enough to permit only the hyperpolarization phase to be visible. The combination of these two local potential changes could give upon the resting membrane potential vacuolar emptying potentials similar to those reported. The positive potential recorded when the artificially enlarged vacuole is impaled would fit into this scheme. In addition to being due to differences in polarity between vacuole and cytoplasm the hyperpolarization could be connected to activity of the contractile mechanism.

The sensitivity of the membrane to basic drugs and cations has been stressed by Brandt (1958), Schumaker (1958) and Chapman-Andresen (1959) working with pinocytosis inducers. Alcian blue, carrying up to four positive charges (Scott *et al.* 1964) is a strong inducer of pinocytosis and has been shown to be adsorbed to the outer surface of the cell membrane. These findings may indicate that the membrane of the amoeba carries negative charges on its surface. In our experiments only inducers of pinocytosis, cations and predominantly positively charged molecules produced membrane depolarization. This may suggest that neutralization of membrane charges is responsible for the permeability increase and subsequently the depolarized membrane undergoes the morphological changes characterizing pinocytosis. The similarity in magnitude of depolarization and decrease of membrane resistance during vacuolar emptying and application of alcian blue gives rise to the suggestion that during induction of pinocytosis neutralization of negative charges of the mucopolysaccharide layer from an electrophysiological point of view would reduce the complex outer membrane to a unit membrane similar to that surrounding the contractile vacuole.

It is a pleasure to thank Dr. Cicily Chapman Andresen and Professor Heinz H[illegible]ter at the [illegible]

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References

BINGLEY, M. S. and C. M. THOMPSON. Bioelectrical potentials in relation to movement in amoebae. *J. theoret. Biol.* 1962. 2. 16–32.
BINGLEY, M. S. Methods for the measurement of bioelectric potentials in amoebae. *Exp. Cell Res.* 1964. 34. 266–282.
BRANDT, P. W. A study of the mechanism of pinocytosis. *Exp. Cell Res.* 1958. 15. 300–313.
CHAPMAN-ANDRESEN, C. Pinocytosis of inorganic salts by *Amoeba Proteus*. *C. R. Lab. Carlsberg* 1958. 31. 77–92.
CHAPMAN-ANDRESEN, C. The effect of pH on the pinocytosis inducing properties of salts and proteins. Soc. Exptl. Biol. Joint British Dutch Scandinavian Meeting. Copenhagen 1959.
CHAPMAN-ANDRESEN, C. Studies on pinocytosis in amoebae. *C. R. Lab. Carlsberg* 1962. 33. 73–264.

CHAPMAN ANDRESEN C in methods in Cell Physiology edited by D M Prescott Chapt 14 New York 1964

CHAPMAN ANDRESEN C and D A T DICK Sodium and bromine fluxes in the amoeba *Chaos Chaos C R Lab Carlsberg* 1962 *32* 445—469

FATT P and B KATZ An analysis of the end plate potentials recorded with an intracellular electrode *J Physiol (Lond)* 1951 *115* 320—370

GREIDER M H W J KOSTIR and W J FRAJOLA Electrone miscroscopy of *Amoeba proteus J Protozool* 1958 *5* 139—146

KLEIN R L Effects of active transport inhibitors on K movement in *Acanthamoeba sp Exp Cell Res* 1964 *34* 231—238

KOEFOED-JOHNSEN V and H H USSING The nature of the frog skin potential *Acta physiol scand* 1958 *42* 298—308

LING G and R W GERARD The normal membrane potential of frogsartorius fibers, *J cell comp Physiol* 1949 *34* 383—412

PRESCOTT D M Mass and clone culture of *Amoeba proteus* and *Chaos Chaos C R Lab Carlsberg* 1956 *30* 1—12

RIDDLE J Studies on the membrane potential of *Pelomyxa carolinensis Exp Cell Res* 1962 *26* 158—167

RUSTAD R C Molecular orientation at the surface of amoebae during pinocytosis *Nature (Lond)* 1959 *183* 1058—1059

SCHATZMANN H J Herzglycoside als Hemmstoffe fur den aktiven Kalium und Natrium Transport durch die Erythrocytenmembran *Helv physiol pharmacol acta* 1953 *11* 346—354

SCHMIDT NIELSEN B and C R SCHRAUGER Amoeba proteus Studying the contractile vacuole by micropuncture *Science* 1963 *139* 606—607

SCHUMAKER V N Uptake of protein from solution by *Amoeba proteus exp Cell Res* 1958 *15* 314—331

SCOTT [illegible]

YAMAGUCHI T J Studies on the modes of ionic behaviour across the ectoplasmic membrane of *Paramecium* I Electric potential differences measured by the intracellular microelectrode *Fac Sci Univ Tokyo* 1960 *8* 573—591

Acta physiol. scand 1966. 66. 406—409

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Storage and in vitro Release Rate of Catecholamines from Granules Isolated from Preaortal Paraganglia and Adrenals of Newborn Rabbits

By

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Abstract

Brundin T *Storage and in vitro release rate of catecholamines from granules isolated from preaortal paraganglia and adrenals of newborn rabbits* Acta physiol scand 1966 66 406—409 — Catecholamine containing granules from paraganglia and adrenals were isolated by homogenization and high speed centrifugation On incubation at 37° C in potassium phosphate buffer pH 7 4 the rate of spontaneous release from paraganglionic granules was similar to that of adrenomedullary granules with a half time of 30 min These similarities between granules from the two organs are discussed in view of the different hormone contents in paraganglia noradrenaline and adrenals adrenaline

The preaortal paraganglia are the main sources for catecholamines CA in fetal and newborn mammals for ref see West 1955 In fetal and newborn rabbits the adrenal medullae of both sides are connected via paraganglionic tissue Kohn 1903 Despite this morphological connection the two parts of this organ complex contain different CA Thus at birth 70 per cent of the adrenomedullary CA consist of adrenaline Roffi 1964 Brundin 1965 a while the paraganglionic part contains almost exclusively noradrenaline (Brundin 1965 b

It has been shown that the CA of the adrenal medulla are bound to subcellular granules (Hillarp Lagerstedt and Nilsson 1953 and others which have also been demonstrated by electron microscopy *in situ* in adrenomedullary cells Lever 1955 and others)

Recently electron microscopic studies of the paraganglia revealed large amounts of cytoplasmic osmiophilic granules also in these organs (Brundin and Nilsson 1965) However, no information seems to be available concerning the characteristics of CA storage mechanisms of paraganglionic granules

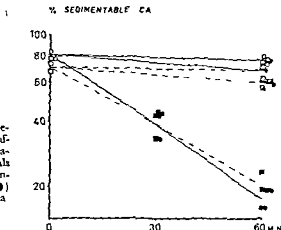


Fig 1 Per cent sedimentable catecholamines after high speed centrifugation of homogenates from paraganglia (□———□) and adrenals (○ —— ○) initially and after incubations at 0° (□, ○), 20° (◪, ◑) and 37° C (■ ●) Ordinate logarithmic scale

In the present study CA storing structures have been isolated from preaortal paraganglia by homogenization and centrifugation The rate of spontaneous CA release from these structures *in vitro* has been determined with special reference to the influence of temperature

Methods

Three experiments were performed Newborn albino rabbits were used After decapitation the preaortal paraganglia and adrenals were removed and separated under a dissection microscope Five paraganglia and ten adrenals were pooled in each experiment The organs were immediately immersed in ice-cold 0 13M potassium phosphate buffer, pH 7 4 The subsequent preparation was performed at ~4° C The pooled organs were separately homogenized by grinding with celite To remove coarse particles the homogenates were centrifuged at 1,000 × *g* for 10 min The low speed supernatants thus obtained from the homogenates of paraganglia and adrenals were both divided into five 2 ml aliquots One of the paraganglionic and one of the adrenal aliquots were immediately centrifuged at 50,000 × *g* for 30 min in a refrigerated centrifuge ("SUPER SPEED 25", MSE) The high speed supernatants were decanted and adjusted to pH 3 5 with 0 4 M perchloric acid The samples were kept frozen until analysis Each high speed sediment was extracted with 1 ml 0 4 M perchloric acid

The *remaining low speed supernatants* of the paraganglia and adrenals respectively were incubated at 0° and 20 C for 60 min and at 37 C for 30 and 60 min before the high speed centrifugation The high speed supernatants and sediments were extracted separately as described above The CA of the extracts were analyzed fluorimetrically according to Euler and Lishajko 1961)

Results

From 70 to 80 per cent of the total CA of the homogenates were found in the high speed sediments from both the adrenal and paraganglionic homogenates Thus the CA of the two organs seemed to be bound to sedimentable structures to a similar extent

After incubations at 0 and 20° C the CA contents of the sediments were only slightly or moderately reduced However, after incubation at 37° C the CA contents of the sediments were decreased while corresponding increases were observed in the supernatants Half of the initially bound CA was recovered from the supernatants after 30 min at 37° C (Fig 1)

Discussion

Cytoplasmic osmiophilic granules have recently been demonstrated in large amounts in paraganglia of newborn rabbits by electron microscopy (Brundin and Nilsson 1965) These granules were of the same size as those found in adrenomedullary cells from the same animals but differed in shape in that the paraganglionic granules showed smaller electron dense cores than those of the adrenal medulla It seems highly probable that the CA-binding structures described in the present study are identical with the granules observed

The present results show that the rate of spontaneous CA release *in vitro* from the paraganglionic granules is similar to that of the adrenomedullary granules obtained from the same animal and under identical experimental conditions

In rabbits the adrenal medulla is derived from the abdominal paraganglia (Kohn 1903) and at birth both adrenal medullae are still connected via paraganglionic tissue However, the paraganglia contain almost exclusively noradrenaline while the adrenals contain mainly adrenaline in newborn rabbits This indicates that the paraganglia are devoid of methylating capacity According to Kirshner and Goodall (1957) adrenaline is formed outside the granules in adrenomedullary cells Thus the difference in hormone content is probably due to extragranular factors

During the fetal development no appreciable quantities of adrenaline have been obtained from the rabbit adrenals until the 20th day of gestation (Brundin 1965 a) At about the same stage of development the contact is established between the abdominal chromaffin tissue and the adrenal cortex (Kohn 1903) These findings suggest a functional relationship between the cortical tissue and the methylation of noradrenaline to adrenaline — In adult animals a possible cortical influence upon the medullary hormone content has previously been suggested (Shepherd and West 1951 and others) Lempinen (1964) showed that administration of adrenocortical hormones to newborn rats preserved the chromaffin reaction in the abdominal paraganglia which in these animals normally disappears rapidly after birth

The present results show similarities between paraganglionic granules containing mainly noradrenaline and adrenomedullary granules containing mainly adrenaline Functional studies on the development and localization of methylating factors necessary for adrenaline formation are in progress

This study has been supported by a grant from Expressen s Prenatal Research Fund

References

BRUNDIN T Catecholamines in adrenals from fetal rabbits *Acta physiol scand* 1965a 63 509—510

BRUNDIN, T, Catecholamines in the preaortal paraganglia of fetal rabbits *Acta physiol s and* 1965b 64 287—288

BRUNDIN T and S L G NILSSON Osmiophilic granules in preaortal paraganglia from newborn rabbits *Acta physiol scand* 1965 65 287—288

EULER U S v and F LISHAJKO Improved technique for the fluorimetric estimation of catecholamines *Acta physiol scand* 1961 *51* 348—355

HILLARP, N Å, S LAGERSTEDT and B NILSON, The isolation of a granular fraction from the suprarenal medulla, containing the sympathomimetic catechol amines *Acta physiol scand* 1953 *29* 251—263

KIRSHNER, N and McC GOODALL, Formation of adrenaline from noradrenaline *Fed Proc* 1957 *16* 73

KOHN, A, Die Paraganglien *Arch mikr Anat* 1903 *62* 263—365

LEMPINEN M Extra adrenal chromaffin tissue of the rat and the effect of cortical hormones on it *Acta physiol scand* 1964 *62* Suppl 231

LEVER J D, Electron microscopic observations on the normal and denervated adrenal medulla of the rat *Endocrinology* 1955 *57* 621—635

Ro[illegible]

S[illegible]

WEST, G B, The comparative pharmacology of the suprarenal medulla *Quart Rev Biol* 1955 *30* 116—137

Acta physiol. scand. 1966. 66. 410–418

From the Department of Physiological Chemistry, University of Lund, Sweden

Intestinal Dipeptidases

III. Characterization and Determination of Dipeptidase Activity in Adult Rat Intestinal Mucosa

By

Lars Josefsson and Tor Lindberg

Received 30 September 196[illegible]

Abstract

Josefsson L. and T. Lindberg. *Intestinal dipeptidases III. Characterization and determination of dipeptidase activity in adult rat intestinal mucosa.* Acta physiol. scand. 1966. 66. 410–418. — Five dipeptidase activities in the mucosa of the rat small intestine have been determined and characterized by the aid of a new spectrophotometric assay method. The distribution of the dipeptidase activities along the gastrointestinal tract has been investigated. The activities were low in the stomach, the cecum and the large intestine, while high activities were found along the whole length of the small intestine with the maximum localized to its middle part. The relation of our present findings to previous observations about the rat intestinal dipeptidases has been discussed. Some differences with respect to both their properties and their distribution were observed in comparison with the dipeptidases of the pig intestinal mucosa.

Our information about the digestion and absorption of different nutrients is mainly based on animal experiments, most frequently on the rat. Thus the absorption of the amino acids has been extensively investigated during the last decades by using the rat small intestine (Wilson 1962). It has been demonstrated by Wiggans and Johnston (1959) and Newey and Smyth (1960) that the dipeptides are hydrolyzed during their transfer through the rat intestine. The site of the hydrolysis of the dipeptides is, however, still unknown despite different suggestions made in favour of either intracellular (Newey and Smyth 1962) or extracellular hydrolysis (Dawson and Holdsworth 1962) on the bases of absorption studies. Elucidation of the question would, however, be greatly facilitated if more reliable information about the dipeptidases in the mucosal cells was available. We have therefore started a general investigation of the dipeptidases in homogenate from different intestinal mucosa by the recently devised spectrophotometric assay method (Josefsson 1964). Previous reports in this series (Josefsson and Lindberg 1965 a, b) have described the properties and distribution of different dipeptidases in the small intestine of the pig. The present report describes the properties of five different dipeptidase activities in the rat intestinal mucosa and their distribution

along the gastrointestinal tract of the rat Previous studies of dipeptidases in rat intestinal mucosa using the earlier assay methods (Linderstrom-Lang 1927, Moore and Stein 1948) have been reported by Berger and Johnson (1939) and by Robinson and Shaw (1960)

Materials and methods

Dipeptides

L Alanyl L-proline H_2O (Yeda, Rehovoth, Israel, Lot no ALPR 2) Analysis, found N,13 40 $C_8H_{14}N_2O_3$, H_2O requires N, 13 71,$[\alpha]_D^{26}$—114 5° (c,2 0 in water), found to be chromatographically pure, R_F 0 30 (Whatman no 1, *n* propanol acetic acid water (4 1 1 v/v))

A 0 006 M aqueous solution was used as substrate

L-Alanyl L-glutamic acid glycylglycine, glycyl-L-leucine and glycyl L-valine, previously characterized (Josefsson and Lindberg 1965 a), were used in the same concentrations as reported earlier (Josefsson and Lindberg 1965 a)

Amino acids

The amino acids were all products of Mann Research Labs , New York and tested chromato-

Animals

Male and female albino rats (Sprague — Dawley strain) of a weight varying between 170 and 230 g (mean weight 200 g) were used The rats were fed on a commercial pellet diet Before the removal of the intestine the animals were starved for at least 15 hrs

Preparation of enzyme solution

The rat was anesthetized with ethyl ether and the intestine was promptly removed before death The intestine was cooled in ice The small intestines were all within 80—100 cm of length For

The clear supernatant was used directly as enzyme solution after suitable dilution with redistilled water according to its total nitrogen content

For the investigation of the distribution of the activities along the small intestine mucosa was scraped off from 2 or 4 cm pieces at different levels of the intestine Enzyme solution from the stomach was obtained from homogenate of mucosa taken from its glandular portion. In the cecum and the large intestine the mucosa was first carefully cleaned by a soft cloth to remove luminal content The whole cecum was used for the scrapings while 2 or 4 cm pieces were taken from the middle of the large intestine.

Some experiments were made to test the efficiency of the scraping procedure Assays revealed that less than 10 % of the different activities were left in the residual wall Histological examination of the different preparates also confirmed that the mucosa was effectively scraped off

Assays

The dipeptidase activities were determined by the spectrophotometric method in the same manner as previously described in detail (Josefsson and Lindberg 1965 a) Buffers and metal salt solutions were also the same as previously reported The pH of the digest mixtures were measured before and after the digestion glass electrode

Nitrogen

Total nitrogen was determined by micro-Kjeldahl procedure with mercuric oxide as catalyst

Unit of dipeptidase activity

One unit is defined as the activity hydrolyzing 1 µmole of dipeptide per min at 40° (Josefsson and Lindberg 1965 b

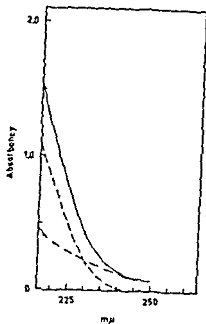


Fig 1

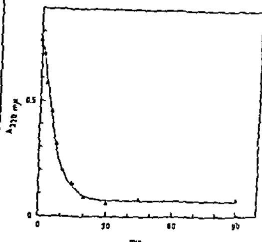


Fig 2

Fig 1 Absorbancy of digest mixtures containing 0 3 μmoles of L-alanyl L-proline (——) and 0 3 μmoles of L-alanine and 0 3 μmoles of L proline (—) at zero time by the standard assay procedure Mucosal extract contained 2 1 μg of nitrogen Phosphate buffer pH 7 0 Difference spectrum (

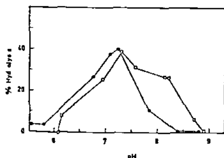
Fig 2 Time-curve for the hydrolysis of L alanyl L-proline by rat intestinal L alanyl L proline dipeptidase added in mucosal extract containing 3 4 μg of nitrogen Phosphate buffer pH 7 0 and 40°

Results

Variation of time and enzyme concentration

The difference spectrum obtained for the new dipeptidase substrate investigated L-alanyl L-proline under the assay procedure is given in Fig 1 The time-course curve determined for its hydrolysis is shown in Fig 2 It closely followed a linear function until about 70 % of the dipeptide was hydrolysed and is therefore comparable to the conditions found valid for L-alanyl L glutamic acid and glycyl L valine A linear function was also obtained when the activity was related to varying amounts of enzyme (total nitrogen added)

The 4 other dipeptidase activities investigated showed time-course curves similar to those obtained in our studies on the pig intestinal mucosa (Josefsson and Lindberg 1965 a) However the glycylglycine dipeptidase activity from the rat intestinal mucosa behaved differently in some respects from what was found when mucosa homogenate from the pig intestine was used The residual activity was rather low because most of our assays had to be done in the presence of 1 nmole of Co^{2+} ions Furthermore, in some experiments we observed that the homogenate did not reduce the absorption of glycyl glycine during the digestion but on the contrary brought about an increase The



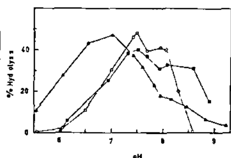


Fig 3 Fig 4

Fig 3 Effect of pH on the rat intestinal dipeptidase activities ● Mucosal extract containing 4 5 μg of nitrogen added to 1 5 μmoles of L-alanyl L glutamic acid 10 min digestion □ Mucosal extract containing 4 1 μg of nitrogen added to 1 5 μmoles of glycyl L-valine 10 min digestion

Fig 4 [illegible] ▲ [illegible] ■ [illegible]

phenomenon was not related to intestines from certain rats as assays of homogenate made from adjacent sections of the same intestine were not disturbed Investigations showed that the increase observed was due to some disturbing substance present in the particular enzyme solution The nature of this substance is still unknown and requires further investigation

Determination of pH-optimum

The different dipeptidase reactions were studied over a pH range from 5 5 to 9 2 by using various phosphate and borate buffer solutions The results obtained are shown in Fig 3 and 4 The following pH optima were observed for L-alanyl L glutamic acid dipeptidase 7 3 for L alanyl L-proline dipeptidase 7 0 for glycylglycine dipeptidase 7 5 (in the presence of 1 mumole Co^{2+} ions) for glycyl L-leucine dipeptidase 7 5 and for glycyl L-valine dipeptidase 7 4 The optima were localized in the phosphate buffer range for all of them and were found to be quite narrow

Influence of metal ions

The effect of the four metal ions on the different dipeptidase reactions when added in various concentrations was studied at their optimum pH The results are presented in Table I where the activity is expressed as the decrease in the absorption per min and mg nitrogen present in the enzyme solution

It is of interest that the Co^{2+} ions which usually form ultraviolet absorbing complexes with some dipeptides especially with glycylglycine Smith 1948 a Josefsson and Lindberg 1965 a) did not show such an interaction in our present studies when the phosphate buffer was used When the borate buffer was used however such a complex was observed in the glycylglycine experiments

TABLE I Effect of bivalent metal ions on the d peptidase activities of rat intestinal mucosa Phosphate buffer Optimum pH

		1 A220 mμ/min/mg N				
Metal ion added	Concentration (nmole)	L-Alanyl L-glutamic acid	L-Alanyl L-proline	Glycyl glycine	Glycyl L-leucine	Glycyl L-valine
Co^2	0	7 64	16 9	0 14	8 09	5 71
	0 1	6 94	16 9	0 20	6 45	4 74
	1	6 60	18 6	0 38	6 49	5 37
	5	3 96	16 4	0 70	5 71	4 43
	10	2 92	18 1	0 79	4 80	5 11
Mn^2	0	7 43	16 9	0 14	8 09	5 71
	0 1	7 29	18 2	0 14	7 51	5 34
	1	5 51	17 4	0 19	6 63	6 26
	5	4 63	16 9	0 18	7 63	5 11
	10	3 89	17 7	0 18	7 09	4 14
Mg^2	0	7 64	21 5	0 14	8 09	5 71
	0 1	8 19	20 9	0 11	7 80	5 76
	1	8 26	21 2	0 14	7 54	5 57
	5	9 03	20 2	0 093	8 80	4 80
	10	8 40	19 9	0 18	8 17	5 89
Zn^2	0	7 43	21 5	0 14	8 09	5 71
	0 1	6 97	20 1	0 15	7 60	6 71
	1	7 43	20 2	0 15	7 06	6 00
	5	7 34	20 1	0 056	7 91	4 51
	10	6 89	20 9	0 076	6 92	4 29

From Table I it is evident that the activity against L-alanyl L glutamic acid was strongly inhibited by Co^2 ions and by Mn^2 ions 60 % and 50 % respectively when 10 nmoles of the metal ions were added Mg^2 ions and Zn^2 ions in contrast were without influence on the hydrolysis

No effect of the metal ions was observed on the activity against L-alanyl L-proline

The Co^2 ions strongly accelerated the hydrolysis of glycylglycine — about 5 fold in the highest concentration while the Mn^2 ions had no definite activating effect Mg^2 ions had no influence while Zn^2 ions inhibited the activity greatly (80 %)

The glycyl L-leucine dipeptidase activity was reduced to about 40 % by Co^2 ions but only slightly by Mn^2 ions and Zn^2 ions Mg^2 ions were also here without influence

The Co^2 ions showed no definite effect on the activity against glycyl L-valine Mn^2 ions and Zn^2 ions on the contrary were both found to inhibit the activity (25 %)

Again Mg^2 ions were without influence

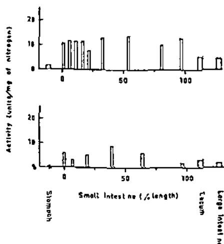


Fig 5

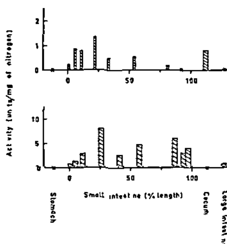


Fig 6

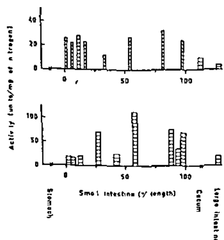


Fig 7

Fig 5 Distribution of L-alanyl L-glutamic acid dipeptidase activity along the gastrointestinal tract in two separate rats Optimum pH and 40°

Fig 6 Distribution of glycylglycine dipeptidase activity along the gastrointestinal tract in two separate rats The activity was measured in the presence of 1 mμmole Co^{2} ions (upper figure) and 5 nmoles Co^{2} ions (lower figure) Optimum pH and 40°

Fig 7 Distribution of glycyl L-leucine dipeptidase activity along the gastrointestinal tract in two separate rats Optimum pH and 40°

Previous investigations (Smith 1948 b, Josefsson and Lindberg 1965 a) have shown that the anions of the buffer affected the influence of the Zn^{2} ions upon the dipeptidase activity Therefore the experiment was repeated by using borate buffer instead of phosphate buffer The results obtained showed that the inhibitory effect of the Zn^{2} ions generally was much greater The activity for L-alanyl L-glutamic acid and for glycylglycine was inhibited completely when 10 nmoles of the Zn^{2} ions were added L-Alanyl L-proline dipeptidase which was not affected at all by the Zn^{2} ions when added in the phosphate buffer system was inhibited about 80 % in the borate buffer system Among the other metal ions only the Mn^{2} ions differed in their effect when

JOSEFSSON L. Determination of dipeptidase activities *Nature* (Lond) 1964 *204* 783—784
JOSEFSSON L and T LINDBERG Intestinal dipeptidases I Spectrophotometric determination and characterization of dipeptidase activity in pig intestinal mucosa *Biochim biophys Acta* (Amst) 1965 *105* 149—161
JOSEFSSON L and T LINDBERG Intestinal dipeptidases II Distribution of dipeptidase activity in the small intestine of the pig *Biochim biophys Acta* (Amst) 1965 *105* 162—166
LINDERSTROM LANG K, Titrimetrisk bestemmelse af amino kvaelstof *C R Lab Carlsberg* 1927 *17* No 4
MOORE, S and W H STEIN Photometric ninhydrin method for use in the chromatography of amino acids *J biol Chem* 1948 *176* 367—388
NEWEY, H and D H SMYTH Intracellular hydrolysis of dipeptides during intestinal absorption. *J Physiol* (Lond) 1960 *152* 367—380
NEWEY H and D H SMYTH Cellular mechanisms in intestinal transfer of amino acids. *J Physiol* (Lond) 1962 *164* 527—551
ROBINSON G B, The hydrolysis of dipeptides by rat intestinal extracts *Biochim biophys Acta* (Amst) 1960 *44* 386—387
[illegible] of rat small [illegible]
SMITH F L. Studies on dipeptidases II Some properties of the glycyl L-leucine dipeptidases of animal tissues *J biol Chem* 1918 b *176* 9—19
WIGGANS, D S and J M JOHNSTON The absorption of peptides *Biochim biophys Acta* (Amst) 1959 *32* 69—73
WILSON, T H *Intestinal Absorption* W B Saunders Co, Philadelphia/London 1962

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From the Department of Biochemistry, University of Aarhus, Denmark

The Excretion of Urate at Various Plasma Concentrations and During Osmotic Diuresis in the Rabbit

By

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Received 1 October 1965

Abstract

MOLLER, J V *The excretion of urate at various plasma concentrations and during osmotic diuresis in the rabbit* Acta physiol scand 1966 *66* 419—426 — The secretory and reabsorptive processes for urate in the rabbit kidney have been studied in experiments in which the plasma urate concentration was varied The curves relating net secretion of urate to the plasma concentration showed an initial dip below zero indicating net reabsorption at low levels, and then rose linearly At very high plasma levels (2—4 µmoles ml) an upper limit for net secretion of urate of 30—35 µmoles/min was demonstrated in 2 expts Osmotic diuresis (induced by infusion of mannitol) did not enhance urate excretion during infusion of urate in contrast to the uricosuric effect of osmotic diuretics previously found at low plasma concentrations where reabsorption of urate prevails The findings may be explained if it is assumed that both secretion and reabsorption are mediated by transport processes which are saturated at high plasma concentrations of urate The extent to which urate is reabsorbed at high plasma concentrations is small relative to the secreted amount of urate The experiments corroborate the view that probenecid in appropriate dosage almost completely inhibits urate secretion

It has been shown in the past years that many substances are both secreted and reabsorbed by the tubular cells of the mammalian kidney Thus the clearance values for for organic acids like salicylate p-hydroxybenzoate and chlorophenol red have been found to reverse from net secretion at low plasma concentrations to net reabsorption at high (Weiner *et al* 1961) The experimental evidence is in agreement with the view that the compounds are secreted by an active process and that they subsequently are reabsorbed by a pH-dependent process (Weiner Blanchard and Mudge 1964) which involves diffusion of the uncharged molecule through the lipid part of the cell membranes (nonionic diffusion)

The renal excretion of urate in man at various plasma levels has been investigated by Berliner *et al* (1950) An apparent T_{max} for reabsorption of about 100 µmoles min

was found but the existence of a tubular secretion of urate (Gutman, Yü and Berger 1959) was not taken into account in these studies. Secretion and reabsorption of urate have been demonstrated in the rabbit but in contrast to the other organic acids mentioned above net reabsorption could only be demonstrated at low ... trations (Poulsen and Praetorius 1954).

The object of the ... reabsorptive process ... of urate at various plasma concentration ... the effect of osmotic diuresis at high plasma concentrations was investigated to find out if back-diffusion of urate plays a significant role for the reabsorption of urate. The results indicate that probably both secretion and reabsorption are mediated by transport processes but that reabsorption is small relative to the secretion of urate at high plasma concentrations.

Methods

The experiments were performed on anesthetized male rabbits weighing 2—4 kg. The anesthesia and surgical procedure are described elsewhere (Møller 196... Infusion of inulin or creatinine at a concentration of 0.5 ... dissolved in physiological saline was started 60—70 min prior to the collection of urine for clearance determinations.

Experiments with rising concentrations of plasma urate. Different levels of plasma urate were in the first experiments obtained by raising the urate concentration in the ... at regular intervals. Urate was added to the ... for clearance determinations. The exc... to a new solution with a ... was also ...

...

... (AH) ... In 4 exp... ml ... at a concen... the whole experimental period. The plasma flow (RPF) in order to deter... experiment.

Experiments with infusion of mannitol. ... effect of osmotic diuresis ... urate excretion was studied in experiments in ... urate was infused at a constant rate ... After collection of urine for 3—5 periods for control determination of urine excretion 10 ml of 20 % mannitol was injected ... the osmotic diuresis sustained by infusion of 10 ... mannitol. After infusion of mannitol for about 15 ... urine was collected for a ... 3—5 periods each having a duration of 5 min.

Calculations. During steady infusion of urate the urate-inulin clearance ratio (C_U/C_{In}) ... $\times C_I$) ... of urate, inulin and creatinine are described elsewhere (Møller 196...).

Results

Urate excretion at varying plasma concentrations of urate. The result of a typical experiment with a continuously rising plasma concentration of urate is shown in Fig. 1. It appears from the graph that there is net reabsorption of urate below 0.03 µmole/ml while at

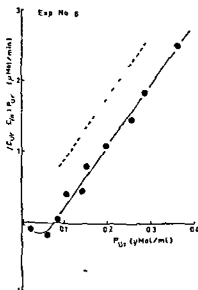


Fig 1

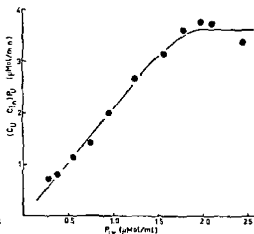


Fig 2

Fig 1 The results of an experiment with a continuously rising concentration of urate in the infusion fluid The dotted lines show how the excretion of urate can be considered as the resultant of a secretion which in the observed range is proportional to the plasma concentration and a reabsorptive process which is saturated at low plasma concentration of urate

Fig 2 Results of an experiment with infusion of large amounts of urate (14—110 µmoles min)

plasma concentrations higher than 0.1 µmole/ml net secretion is evident and the points apparently scatter around a straight line

These findings may be explained if it is assumed that the true secretion of urate is proportional to the plasma urate concentration but is counteracted by a reabsorptive mechanism which is saturated at high plasma concentrations The hypothetical curves for urate secretion and reabsorption are indicated by the dotted lines in the figure

The same results were obtained in three experiments with a continuously rising plasma concentration, and in three experiments employing stepwise increments in the concentration of urate in the infusion fluid The curves relating net secretion to plasma urate concentration showed an initial dip below zero indicating net reabsorption at low plasma levels and then rose linearly at plasma concentrations up to 0.6 µmole/ml

The regression coefficient of the straight line through the experimental points has been calculated by the method of least squares in all of the 7 expts This coefficient (expressed as µmoles min µmoles/ml ml/min) ranged from 7.0 to 17.6 ml min with a mean of 10.6 ml min The correlation coefficient varied between 0.97 and 0.99 which indicates a good correlation between the plasma concentration and the net secreted amount of urate assuming a linear relationship between the plasma concentration and the net secreted amount of urate It appears from the table that the intersection of the straight line with the Y axis is in all instances negative with a mean of — 0.64 µmole min and ranging from — 0.23 to — 1.48 µmole min

TABLE 1 Summary of experiments with rising plasma concentrations of urate

I refers to experiments with stepwise increments in the concentration of urate in the infusion fluid and II to experiments employing a continuously rising concentration of urate in the infusion fluid

Exp no	Type of experiment	Regression coefficient (ml/min)	Intercept with X axis (µmoles/min)	Correlation coefficient
1	I	8 1	−0 44	0 93
2	I	17 6	− 1 03	0 93
3	I	7 0	−0 23	0 98
4	II	9 0	−0 29	0 99
5	II	8 0	− 1 48	0 97
6	II	8 5	−0 66	0 99
7	II	16 3	−0.28	0 93

The experimental results thus confirm the theory of a bidirectional transport of urate characterized by a relatively small reabsorption of urate at high plasma concentrations However no deflection in the curve of net secretion of urate was found in these experiments In 4 expts larger amounts of urate were infused to examine if it was possible to demonstrate a saturation of the secretory system The plasma concentration reached a level of 2—4 µmoles ml in all experiments The results of one of these experiments are presented in Fig 2 It appears from the figure that the secreted amount of urate apparently reaches a maximal value of about 35 µmoles min at plasma concentrations above 2 µmoles ml It may be noted that reabsorption of urate is not detectable in this experiment but this presumably reflects the fact that secretion of urate is much larger than reabsorption of urate at these high plasma concentrations An upper limit for urate secretion of about 30 µmoles min was also found in one experiment, but in 2 expts the secreted amount of urate remained proportional to the plasma concentration of urate although the secreted amount of urate exceeded 30 µmoles min in both instances

It is perhaps possible that the decline in the net secreted amount of urate in 2 of the experiments mentioned above might be due to a diminished supply of urate to the tubules, resulting from a decrease in renal blood flow at the termination of the experiments In order to evaluate this possibility RPF estimated as C_{PAH} at low plasma concentrations of PAH has been measured in 4 expts. Fig 3 shows that in expts a and b no tendency for a fall in C_{PAH} is observed during the course of the experiment, whereas a small decrease in C_{PAH} is evident in expts c and d Similar changes in C_{In} were observed, and C_{PAH} C_{In} ranged from 3 26 to 4 55 mean value of the 4 expts 3 73 As reported elsewhere C_{Ur} C_{Cr} averaged 1 8 during infusion of urate Moller 1966) which presumably means that urate is only partially extracted from the renal plasma also at the end of the experiment. It may therefore be concluded that the tubular load of urate is adequate for the demonstration of a T_{max} value (Smith 1956) The subject is more fully discussed below see *Results*

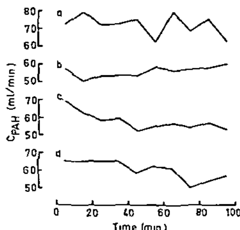


Fig 3 Variations in PAH clearance during the course of 4 expts

The effect of mannitol on urate excretion during infusion of urate It has previously been shown that osmotic diuresis enhances the excretion of urate at low plasma concentrations of urate (Moller 1962) According to the proposed theory only a small part of urate (relative to the secreted amount) is reabsorbed at high plasma concentrations, and so it is of interest to investigate the effect of osmotic diuresis at high plasma concentrations of urate The result of 4 expts in which the effect of mannitol on urate excretion was examined during infusion of urate, is presented in Table II The mean

TABLE II The effect of mannitol on urate excretion C_{Ur}/C_{In} during infusion of urate

Exp no	Infusion of mannitol	No of periods	P_{Ur} (μmoles/l)	C_{In} (ml min)	C_{Ur}/C_{In}	V/C_{In}
8	0	3	98	96	179	02
			(96–101		(167–193)	
	+	4	99±18	120	170± 13	43
9	0	3	221	127	197	03
			211–226)		(196 193)	
	+	5	204±20	147	169± 06	25
10	0	3	163	106	223	09
			155–180		210–233)	
	+	4	231±25	110	221± 05	53
11	0	5	141–20	116	148± 05	26
	+	3	155	118	128	50
			(139–173)		(119–145)	

Abbreviations are as follows P_{Ur} plasma concentration of urate C_{In} = inulin clearance, C_{Ur}/C_{In} = urate-inulin clearance ratio V – diuresis

value for C_{In} was 11.1 ml/min before and 12.4 ml/min after administration of mannitol; by contrast a sharp rise in the diuresis was noted, V/C_{In} increasing from an average value of 0.10 to 0.43. P_{ur} rose from a mean of 156 μmoles/l to 172 μmoles/l. C_{Ur}/C_{In} had a mean value of 1.87 prior to infusion of mannitol and averaged 1.72 after establishment of osmotic diuresis. In expts. 8 and 10 no change in C_{Ur}/C_{In} was detected, whereas a moderate decline was observed in expts. 9 and 11 from 1.97 and 1.48 to 1.69 and 1.28, respectively.

The reason for the decreased values of C_{Ur}/C_{In} in some of the experiments is uncertain. However, it is of interest that no uricosuria occurred in any of the experiments, in contrast to the results previously obtained by infusion of osmotic diuretics at endogenous plasma levels of urate (Møller 1962). This finding suggests that back diffusion of urate only plays an insignificant role for the reabsorption of urate, because one would then expect a uricosuric effect of mannitol both at low and high plasma concentrations of urate.

Discussion

The simultaneous secretion and reabsorption of urate in the rabbit kidney. The excretion of urate at various plasma concentrations and during infusion of an osmotic diuretic (mannitol) has been examined in the rabbit. In agreement with a previous investigation (Poulsen and Praetorius 1954), net reabsorption was found at low and net secretion at high plasma levels of urate. The secretory process appears to be proportional to the plasma concentration over a wide range, but is counteracted by a reabsorptive process having a maximum capacity. The reabsorption is characterized by being more efficient than secretion of urate at low plasma concentrations during infusion of physiological saline, but the reabsorptive system is apparently saturated during infusion of urate.

Osmotic diuretics have previously been shown to enhance urate excretion at low plasma concentrations (Møller 1962). This may be due to either an inhibition of urate reabsorption or a stimulation of urate secretion. The present experiments show that the excretion of urate was practically unaltered during infusion of mannitol at high plasma concentrations of urate, i.e. when the reabsorption of urate is relatively small. It is therefore reasonable to assume that the secretory process is not affected by infusion of mannitol, the uricosuria at low plasma concentrations being due to an inhibition of reabsorption of urate.

The mechanism of urate reabsorption. Due to the intense secretion of urate in the rabbit kidney, it is difficult to draw definite conclusions as to the nature of urate reabsorption. Urate may be reabsorbed either by a transport process or by diffusion from the tubular fluid to plasma. As mentioned in the introductory section, many organic acids apparently are reabsorbed by non-ionic diffusion. The excretion of many of these substances is enhanced by alkalinization of the urine (Weiner *et al.* 1964) and the back diffusion at constant diuresis and pH is presumably proportional to the plasma concentration (Weiner *et al.* 1961). It appears unlikely, however, that urate should be reabsorbed by non-ionic diffusion, since such a process would not be expected to be rate limited. Furthermore, uric acid is not lipophilic, and according to Poulsen and Praetorius

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fluid to plasma was secondary to the active reabsorption of Na However a uricosuric effect of mannitol should then be observed both at low and high plasma concentrations as a consequence of diminished reabsorption of Na^+ under these circumstances. Furthermore reabsorption of urate would not be expected to be rate limited if urate were freely diffusible through the tubular cell membranes

On the basis of these considerations the author regards the existence of a specific mechanism for the reabsorption of urate as the most likely possibility The experiments do not show if such a transport is of an active nature or effected by a carrier-equilibrating" mechanism similar to that which has been demonstrated for the passage of e g uric acid (Lassen 1962) across the human erythrocyte membrane

It may seem curious that at low plasma levels reabsorption of urate is more efficient than secretion of urate during infusion of saline in spite of the fact that much more urate is secreted at high plasma concentrations This finding does not necessarily imply a greater affinity of urate for the reabsorbtive system A c nsequence of urate secretion and reabsorption of water is that the concentration of urat in the tubular fluid is larger than in plasma This will probab y facilitate reabsorption of urate if the reabsorptive system is not saturated i e at low plasma concentrations In this way the uricosuric effect of mannitol at low plasma concentrations may at least in part be explained by the smaller gradients of urate between tubular fluid and plasma which exists under these conditions At high plasma concentrations however the reabsorptive system is saturated with urate whether mannitol is infused or not and in agreement with the experimental findings a uricosuric effect can not be demonstrated

Secretion of urate The experiments with infusion of moderate amounts of urate indicate that the secreted amount under these conditions is probably proportional to the plasma concentration After administration of large amounts of urate an upper limit for urate secretion was found in 2 of 4 experiments but in these experiments a fall in C_{In} of the order of 15—20 % was noted In agreement with Schou (1942 and Forster (1947) a correlation between the glomerular filtration rate and RPF was found in the experiments with infusion of PAH and it is therefore reasonable to asume that a fall in RPF has occurred in these 2 expts The question then arises if the decline in the secreted amount of urate might be due to a diminished blood flow through the kidneys at the end of the experiments However in 2 of the 7 expts with infusion of smaller amounts of urate a fall in C_{In} was observed which was not accompanied by a similar decrease in the net secreted amount of urate It is possible that changes in RPF only slightly affect urate secretion In the case of PAH Harth Kreienberg and Lutz (1959) have found that the extraction of PAH is dependent on RPF thus tending to minimize the effect of changes in RPF on the secreted amount of PAH

Studies on the urate secretion in the chicken Berger Yu and Gutman 1960 and Dalmatian dog (Yu *et al* 1960 have shown that this process is inhibited by PAH Platts and Mudge (1961 have found an intracellular accumulation of urate in rabbit kidney slices. The uptake of urate was depressed by PAH diodrast and probenecid These findings suggest that urate is secreted by a similar mechanism as for other organic acids l ke PAH and diodrast and therefore it is of interest to compare the excretion of urate with these substances n vivo

During steady infusion of urate in doses insufficient to saturate the secretory system C_U/C_{Cr} averages 1 8 (o lsen and Praetorius 1954 Möller 1966 whereas the clearance ratios for diodrast s about 5 Berlin 1962 and for PAH a mean value of 3 7 was found in the presen study The affinity of urate for the secretory system is thus

smaller when compared with these organic acids. However the amount of urate which may be secreted at high plasma concentrations appears to be of a similar magnitude to those of diodrast (Josephson *et al* 1954) and PAH (Foulkes and Miller 1959).

It has been reported in another paper (Møller 1966) that C_U/C_{Cr} during infusion of urate decreased to 0.8—0.9 after administration of 100 mg probenecid/kg. It was suggested that probenecid is able to inhibit almost completely the secretion of urate in the rabbit whereas reabsorption of urate was only slightly affected if at all. If urate secretion is abolished after infusion of probenecid while a small but continuing reabsorption of urate still takes place C_U will be depressed to a little below the glomerular filtration rate. The present findings are therefore in agreement with the view that secretion of urate may be arrested by probenecid.

References

[illegible] 254

BERLINER R. W., J. G. HILTON, T. F. YU and T. J. KENNEDY. The renal mechanism for urate excretion in man. *J. clin. Invest.* 1950. *29*. 39[illegible]—401.

FORSTER, R. P. An examination of some factors which alter glomerular activity in the rabbit kidney. *Amer. J. Physiol.* 1947. *150*. 523—533.

FOULKES, E. C. and B. F. MILLER. Transport of p-aminohippurate from cell to lumen in kidney tubule. *Amer. J. Physiol.* 1959. *19[illegible]*. 83—85.

GUTMAN, A. B., T. F. YU and L. BERGER. Tubular secretion of urate in man. *J. clin. Invest.* 1959. *38*. 1778—1781.

HARTH, O., W. KREIENBERG and J. LUTZ. Der Einfluss von Hydramie und Nierendurchblutung auf die renale Extraktion der p-Aminohippursäure. *Pflügers Arch. ges. Physiol.* 1959. [illegible] 174—183.

JOSEPHSON, B., A. GRIEG, G. KAKOSSAIOS and J. KALLAS. Renal tubular excretion from high plasma levels of para aminohippurate (PAH) and diodrast (D) in unanaesthetized rabbits. *Acta physiol. scand.* 1954. *30*. 11—21.

LASSEN, L. V. *Urinsyretransport gennem humane celmembraner.* Kbh. 196[illegible].

MØLLER, J. V. The effects of D- and L-lactate and osmotic diuresis on uric acid clearance in the rabbit. *Acta physiol. scand.* 196[illegible]. *5[illegible]*. 30—36.

MØLLER, J. V. Clearance experiments on the effect of probenecid on urate excretion in the rabbit. *Acta pharmacol.* Kbh. 1966. In press.

PLATTS, M. M. and G. H. MUDGE. Accumulation of uric acid by slices of kidney cortex. *Amer. J. Physiol.* 1961. *200*. 387—391.

POULSEN, H. and E. PRAETORIUS. Tubular excretion of uric acid in rabbits. *Acta pharmacol.* (Kbh.) 1954. *10*. 371—378.

SCHOU, P. Eksperimentelle undersøgelser over sulfa diuresen. Kbh. 194[illegible].

SMITH, H. W. *Principles of renal physiology.* Oxford University Press 1956.

WEINER, I. M., K. C. BLANCHARD and G. H. MUDGE. Factors influencing renal excretion of foreign organic acids. *Amer. J. Physiol.* 1964. *207*. 953—963.

WEINER, I. M., K. D. GARLID, J. A. ROMEO and G. H. MUDGE. Effects of tubular secretion and reabsorption on titration curves of tubular transport. *Amer. J. Physiol.* 1961. *[illegible]*. 393—399.

YU, T. F., L. BERGER, S. KUPFER and A. B. GUTMAN. Tubular secretion of urate in the dog. *Amer. J. Physiol.* 1960. *199*. 1199—1204.

Acta physiol. scand. 1966. 66. 427—436

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Effects of Sympathetic Vasoconstrictor Fibres, Noradrenaline and Vasopressin on the Intestinal Vascular Resistance during Constant Blood Flow or Blood Pressure

By

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Abstract

Dresel, P and I Wallentin *Effects of sympathetic vasoconstrictor fibres noradrenaline and vasopressin on the intestinal vascular resistance during constant blood flow or blood pressure* Acta physiol scand 1966 66 427—436 *The effects of stimulating vasoconstrictor fibres and infusing noradrenaline* and vasopressin on intestinal vascular resistance were studied in cats using either constant flow or constant pressure The responses to stimulating constrictor fibres and to infusing noradrenaline were essentially the same whether pressure or blood flow was kept constant and the earlier described autoregulatory escape' was of about the same magnitude during both these conditions The results further support the hypothesis that a redistribution of blood flow takes place within the intestinal wall and the combined evidence suggests an increased submucosal flow at the expense of the mucosa The similarities between infusing noradrenaline and stimu [illegible] ngs and the α receptors [illegible] is supported by the fact [illegible] sustained vasoconstrictor [illegible] autoregulatory escape [illegible] nt pressures are pointed out It is further emphasized that both these phenomena are very often diminished distorted or even totally suppressed by the introduction of an artificial pump system

Recent reports from this laboratory have described the response of the intestinal vessels to prolonged sympathetic stimulation (Folkow *et al* 1964 a, b) It was shown that the intestinal vascular resistance after an initial often very pronounced, increase, returned towards the prestimulatory level within a few minutes despite continued stimulation of vasoconstrictor fibres There was a poststimulatory reactive hyperemia This 'autoregulatory escape of the resistance vessels from the constrictor fibre influence was supposed to be a local adjustment since the veins remained constricted and the capillary

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surface area open to flow remained considerably reduced throughout the period of stimulation, even in cases where the increased neurogenic resistance was entirely abolished These findings, supported by microscopical studies of preparations with India ink injected into the intestinal vascular bed before and during stimulation of the vasoconstrictor fibres led to the hypothesis that the constrictor fibres induce a redistribution of blood flow from the mucosa to submucosal structures It was suggested that this redistribution involves an opening of submucosal low resistance channels and the possible involvement of the shunt-like vessels, described by Spanner (1932) was discussed. It was emphasized, however, that a neurogenic reduction of mucosal blood flow will cause a local metabolite accumulation, which by counteracting the mucosal vasoconstriction may contribute to the autoregulatory escape

The present report describes experiments made to determine the following points concerning autoregulatory escape 1 Is the redistribution of blood flow a response to decreased flow *per se*, i e would it not occur if total blood flow was maintained constant by means of pump perfusion? 2 Is the observed response due to a distribution in the constrictor fibre supply to the intestinal vessels, where e g the submucosal vessels largely lack such a nerve supply? If so, the characteristic resistance response would appear only when intestinal vasoconstriction is induced by the vasomotor nerves and not when constrictor agents are given in the blood stream 3 If however the response occurs also under the latter circumstances, is it then confined only to catecholamines because of a specific vascular distribution of *a* receptors or does it occur also when overall constrictor agents like vasopressin are given?

Methods

52 cats of either sex weighing 1 7—3 8 kg were used Anesthesia was induced with ether and maintained either by i v administration of 25—30 mg / kg pentobarbitone sodium of chlora [illegible] ...hing the arterial [illegible] de- [illegible] 0 g [illegible] nu- [illegible] rop [illegible] recorder operated an ordinate writer recording on a smoked paper kymograph The venous [illegible] of the jugular veins [illegible] ed through either a [illegible] The arterial circuit [illegible] ed to vary Special [illegible] leave the adjacent vasomotor nerves to the intestine undisturbed in those experiments where the splanchnic nerves were to be stimulated Further in these cases the two adrenal medullae were selectively denervated by ligation of the thin nerves entering the glands from the coeliac plexus The intestinal vessels could be entirely denervated in the course of the experiments when the effects of drugs were to be studied either by cutting all tissue along the intestinal pedicle except the artery and vein or in early experiments by cutting both splanchnic nerves

In another group of the animals especially in some cases when the effects of stimulating vasoconstrictor fibres were to be studied the superior mesenteric arterial connections were left entirely intact the intestine being supplied in the normal way by blood directly from the heart In these preparations an arterial clamp was placed around the aorta just below the diaphragm so that the pressure head to the intestine could be kept constant when desired

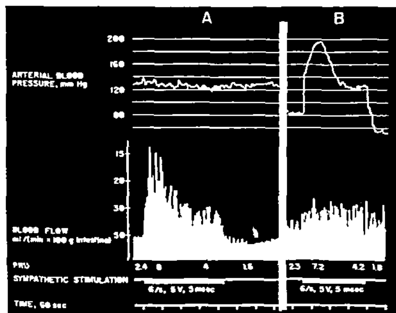


Fig 1 C 2 9 k D
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proximally to the mesenteric artery
A. Arterial inflow pressure to the intestine is kept constant before during and after splanchnic nerve st mulation (constant pressure autoperfus on
B Intestinal blood flow kept roughly constant before during and af er splanchnic nerve stimulation, by a continuous adjustment of the aort c clamp as guided by the blood flow recording (constant flow autoperfusion)
Note that the changes in intestinal vascular resistance during and after stimulating the splanchnic nerves are essentially the same whether pressure (A or flow B are kept constant both cases show peak vasoconstriction, autoregulatory escape and poststimulatory hyperemia of approximately equal magnitudes

It could also allow constant flow perfus on if the arterial pressure was first raised by a carotid occlus on up to some 250 mm Hg In these cases both vagal nerves were cut in the neck. Then, by means of a screwclamp the pressure head to the ntestine was initially kept at the level of 80 mm Hg before the splanchnic stimulat on was started. By manually adjusting the clamp the blood flow through the intestine could of en be kept nearly constant during the period of splanchnic nerve stimulation. Thus gu ded by the cont nuously recorded drop rate, in estinal blood flow could be kept essentially constant This was cons dered to be a valuable type of control experiment, as the introduction of the pump circu t often drast cally reduced vascular reactivity see below

The splanchnic nerves on both sides were cut just beneath the diaphragm Only the left nerve was stimulated n some experiments. St mulation of both nerves in the other experiments did not result in any d fference in the nature of the responses A Grass Model S-4 stimulator was connected through an isolat on un t to b polar silver electrodes surrounding the nerves. Stimulus parame ers were 10 V 3—4 m llisec duration and frequencies of 4—16 sec In some cases the postgangl on c nerve fibres were st mulated by placing the electrode on the nerve plexus surrounding the superior mesenteric artery As the pos ganglionic fibres have a higher

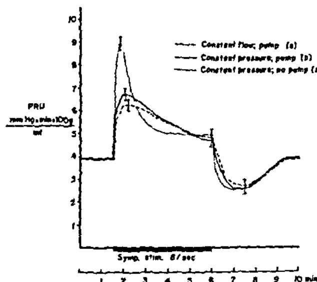


Fig 2 Cat 3 kg Bilateral splanchnic stimulation. In curves a and b an artificial pump system delivered the ...

Note the similarities in flow resistance changes between curves a and b where the pump delivers a constant flow (a) and a constant pressure (b) Note also how both these responses, involving an artificial pump, are considerably distorted as compared with the events in curve c where the animal's heart instead of the artificial pump system, delivers the intestinal blood flow via the same polyethylene tubing

threshold the voltage was raised in these cases to 10—12 V The vascular responses to stimulation were generally the same as when preganglionic stimulation was performed

lamp Drying of the tissues was prevented by saline-soaked plastic-covered gauze pads during the preparation and by closing the abdomen whenever possible

connection to the superior mesenteric artery A screw clamp on the pump bypass was used to maintain the perfusion pressure constant in those experiments in which the flow was permitted to vary

Results

1. *Effects of sympathetic vasoconstrictor fibre stimulation and infusion of noradrenaline*

The effect of prolonged nerve stimulation on the blood flow under conditions of constant perfusion pressure has been illustrated in previous publications (Folkow *et al*

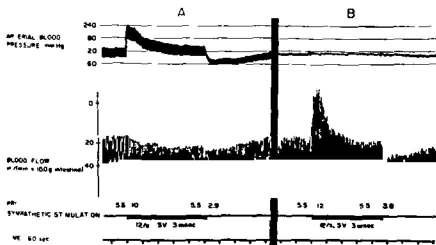


Fig 3 [illegible]
poststimulatory hyperemia are very similar in the two cases

1964 a, b) The effects of nerve stimulation under conditions of constant arterial inflow are substantially the same as is shown in Fig 1, 2 and 3

In the experiments shown in Fig 1 both splanchnic nerves were prepared for stimulation and both adrenal glands were selectively denervated The vagal nerves had been cut and the carotid arteries were clamped during the two sequences shown in the figure so as to create a high arterial pressure However with the aid of a clamp around the aorta, placed just below the diaphragm, the perfusion pressure to the intestine could be adjusted to any desired level In the left hand section of the figure arterial pressure is thus kept essentially constant around 125—130 mm Hg before, during and after a period of splanchnic stimulation at 6 impulses per second After an intense initial vasoconstriction the typical 'autoregulatory escape' is seen, followed by a fairly prolonged poststimulatory hyperemia The calculated flow resistance changes are also given in PRU values In the right hand section, performed about 10 min later the same splanchnic stimulation was repeated but now attempts were made to keep blood flow as constant as possible by allowing the perfusion pressure to change in phase with the resistance changes For this purpose the arterial pressure was initially kept as low as 80 mm Hg, and when the splanchnic stimulation was started the clamp was steadily adjusted to keep blood flow as constant as possible It was, however impossible to avoid a slight decrease in blood flow as the animal did not quite manage to keep an arterial pressure level high enough to match the increase in intestinal blood flow resistance In any case, even if flow was not quite constant it decreased only some 25—30 % and was kept at this level throughout the stimulation period It is seen from the blood pressure recording and the calculated resistance values that almost the same extent

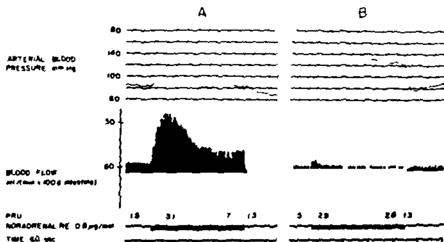


Fig 4 Cat 3.2 kg Same arrangements as in Figs 2 and 3 Intestinal blood vessels denervated Constant infusion of noradrenaline during constant pressure perfusion (A) and constant flow perfusion (B) *Note that the diffuse blood borne distribution of the adrenergic transmitter induced about the same pattern of vascular response as when the transmitter is locally released at the constrictor nerve endings* (compare Fig 1)

of 'autoregulatory escape occurred during this 'constant flow condition as when a 'constant pressure perfusion was maintained

Fig 2 illustrates what very often happens with the 'autoregulatory escape by introduction of an artificial pump system In this experiment the intestine was supplied with arterial blood from the carotid artery via a polyethylene tube system It was now possible during repeated vasoconstrictor fibre stimulations to keep either the inflow pressure constant with a screw-clamp (curve c) or blood flow constant by means of a Harvard perfusion pump (curve a) It was also possible to let the pump deliver the inflow and match the pump rate to the changing peripheral resistance during stimulation to keep the inflow pressure constant (curve b) It is important to note that the three stimulation periods constituting each one of the three curves a b and c were not done after each other but at random This excludes the time factor and a possible influence of the polyethylene tube systems as this was used during the whole experiment as causes to the differences shown in Fig 2 When the heart delivers the flow (curve c) the increasing resistance during stimulation of vasoconstrictor fibres is very pronounced but declines rapidly within 1 min Fig 1 B shows the same type of autoregulatory escape' when the heart is delivering a 'constant flow When the pump is involved (curves b and c in Fig 2 the peak responses are only about 50 % of the peak in curve c The resistance then declinenes sluggishly but no stable plateau is reached during the about 6 min periods of stimulation The only difference in experimental procedure between curve b and c is that its source of pressure in b is the Harvard pump and in c the animal's own heart

Fig 3 illustrates that it was sometimes but seldom possible to maintain an almost normal vascular reactivity when the artificial pump system was used

Intraarterial infusion of noradrenaline into the intestinal segment in doses of 0 4 to

16 μg/min caused abrupt vasoconstriction which was followed by 'autoregulatory escape Fig 4 shows that the intestinal vascular resistance is increased to approximately twice the control value when 0 8 γ noradrenaline is infused per minute during perfusion at constant pressure (left panel) The vascular resistance starts to fall towards the control value within 60 sec. A 'steady state' level of resistance, only slightly above the control value, is reached and maintained throughout the remainder of the infusion Termination of the infusion was followed by some reactive hyperemia The same noradrenaline infusion performed during constant flow perfusion (right hand panel), induces essentially the same changes in vascular resistance, only with slightly less pronounced 'escape' from the initial vasoconstriction

The difference between the 'steady state' resistance and the control resistance was quite variable in the experiments where noradrenaline was infused but usually the 'steady state resistance was only some 10—25 % greater than the control, even though the initial vasoconstrictor response could be very pronounced In some of the experiments the 'steady state' resistance was the same or even less than that of control whereas in a few cases where the preparation had deteriorated 'autoregulatory escape' was only slight or even absent Some oscillation of resistance prior to the establishment of the steady state was also seen The reactive hyperemia was also quite variable both in extent and in duration In general, it seemed to be correlated with the degree of 'autoregulatory escape', being greater whenever the escape phenomenon was pronounced In those few experiments where 'autoregulatory escape' did not occur, reactive hyperemia was also absent

The response to infusions of noradrenaline is thus generally the same as that to stimulations of the intestinal vasoconstrictor fibres as shown above and illustrated in an earlier publication (Folkow *et al* 1964 a b) However, in most cases the onset of 'autoregulatory escape' seems to be definitely more rapid and the slope of the return toward control values of peripheral resistance steeper during constrictor fibre stimulation than during infusion of noradrenaline

2 *Effects of vasopressin*

Vasopressin was used for comparison with the noradrenaline effects as this hormone is considered to act as a stimulant of the resistance vessels in all organs studied so far Attempts were also made to utilize angiotensin but as the intestinal blood vessels very rapidly developed tachyphylaxis to this vasoconstrictor agent it could not be utilized for the present purpose The response of the intestinal resistance vessels to infusions of vasopressin in doses of 0 024—0 08 IU / ml was entirely different from that observed during infusions of the noradrenaline Significant autoregulatory escape from the vasoconstrictor influence of vasopressin was not seen Fig 5 shows the experiment which showed the most pronounced escape noted in the 12 experiments with vasopressin Both at constant pressure and at constant flow perfusion the increase in vascular resistance was well maintained in practically all the cases throughout the period of vasopressin infusion This was the case also when the intestine was perfused in the normal way from the heart with no pump interference on vascular reactivity Return to control conditions after termination of the infusion was often considerably slower than the recovery seen in Fig 5 No reactive hyperemia was observed in any of the animals even when the blood flow had been reduced for a considerable period by the action of the hormone under conditions of constant perfusion pressure To ascertain that the sustained increases in vascular resistance were not at least in part, due to a vasopressin

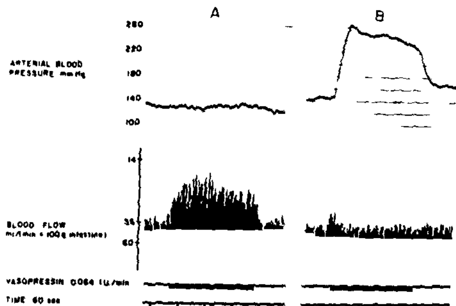


Fig. 5. Cat 2.7 kg. Same arrangements as in Fig. 4. Constant infusion of vasopressin during constant pressure perfusion (left) and constant flow perfusion (right). The figure is taken from the one out of twelve experiments where the vasopressin vasoconstrictor effect on the intestinal vessels shows the greatest extent of decline during the course of the constant infusion. Note that neither any typical autoregulatory escape nor any reactive hyperemia is seen in spite of the fact that total blood flow is much more reduced than during infusion of noradrenaline or stimulation of vasoconstrictor fibres.

induced intense contraction of the intestinal wall causing a mechanical blood flow obstruction, the intraluminal pressure or volume was recorded in some of the experiments. If anything, the doses of vasopressin used relaxed the intestinal smooth muscles, implying that no mechanical blood flow obstruction was involved (*cf.* also Lexter *et al.* 1964).

Discussion

In the present study it has been shown that the intestinal resistance vessels exhibit "autoregulatory escape" from the effects of stimulating vasoconstrictor fibres almost as readily when total intestinal blood flow is maintained constant as when the initial increase in resistance causes a decrease in flow. Therefore, the answer to the first question, stated in the introduction, is that the neurogenic reduction of the *total* blood flow *per se* can not be the initiating factor in "autoregulatory escape". On the other hand the maintenance of constant blood flow through a vascular bed with alternative channels does not necessarily prevent the accumulation of vasodilator metabolites in some regions. The influence of constrictor fibres to some area, e.g. the mucosa, may well be intense enough to reduce its blood flow considerably, which would tend to accumulate metabolites in this area. This, in turn, would tend to restore partially the neurogenically reduced blood flow, a phenomenon which can be expected to contribute to the autoregulatory escape. It is, however, a prerequisite for the metabolite accumulation in the

mucosa that there is an initial mucosal flow reduction When therefore total blood flow is kept unchanged during constant flow perfusion there must be a compensatory flow increase through low resistance vessels in other sections or the tissue, where either the adrenergic fibres do not constrict the vessels of where they actually might bring about lowering of the resistance This blood flow redistribution, as supported by a secondary metabolite accumulation in the mucosa, may satisfactorily explain both the 'autoregulatory escape' and the poststimulatory hyperemia

The actual events taking place when flow increases in such presumed low resistance channels are not known Their widening might be a purely passive phenomenon caused by a regional transmural pressure increase in a very distensible vessel Such a local pressure increase may indeed occur, even if central arterial pressure is kept unchanged provided that the low resistance channels branch off from the intestinal precapillary resistance section just upstream to the site where the neurogenic obstruction of the branches supplying the mucosa takes place Another possibility is that these 'low resistance channels' are actually controlled by adrenergic fibres According to Spanner (1932) the submucosal shunt like vessels have longitudinal smooth muscles In case these are contracted by the direct influence of adrenergic fibres a vascular shortening would be brought about, possibly even some widening This might actually reduce their resistance, at least when they are exposed to a reasonable transmural pressure (Folkow *et al* 1964 b)

These alternatives — both a passive opening of low resistance channels induced by a raised pressure or an active opening of nerve controlled vessels — can explain a redistribution of blood flow but can hardly explain why it should be a delay of 1—2 min before the redistribution begins It seems likely that a passive opening would operate in pace with the increasing resistance It is also difficult to understand that a direct nerve effect should take 1 min or even more before any change is noticeable It then seems more likely that a local vasodilator mechanism gradually builds up and produces a slowly developing relaxation of some section of the submucosal vasculature

The answer to the second question stated in the introduction is that the complex vascular adjustments induced by stimulating constrictor fibres appear to be closely mimicked by those induced by noradrenaline infusion In case the neurogenic response is a mere consequence of a highly selective constrictor fibre distribution to some key points in the vascular resistance section it then follows that the adrenergic α receptors in all probability show a tendency to be similarly restricted in distribution Histological findings of Norberg (1965) suggest that such a selective distribution of constrictor fibres in the intestine exists as these fibres appear to be mainly confined to basal parts of the mucosal arterial tree

The answer to the third question stated in the introduction is that not all vasoconstrictor agents produce the same differentiated pattern of intestinal vascular response as is induced by noradrenaline when released at the vasoconstrictor nerve endings or supplied via the blood stream Vasopressin infusion produced the well known sustained increase in intestinal vascular resistance for ref see Tsakiris Haemmerli and Buhlmann, 1964) both at constant pressure and constant flow perfusions with no signs of flow redistribution It thus seems as if a specific distribution of the adrenergic α receptors within the intestinal arterial tree is at least one of the prerequisites for the events seen when the intestinal vascular bed is exposed to increased sympathetic activity This by no means denies the possibility that the autoregulatory escap and the poststimulatory hyperemia are to a great extent also dependent on a local adjust

ment of a particular vascular compartment, which becomes secondarily involved after the adrenergic constrictor effect is initiated. Such a possibility is supported by the fact that many similarities exist between the 'autoregulatory escape' from constrictor influences and the vascular adjustments occurring upon changes in arterial pressure. For instance, capillary pressure remains surprisingly constant in both situations. Furthermore, we have observed repeatedly that 'autoregulatory escape' and autoregulation to pressure changes diminish *together* as the preparation deteriorates, where incidentally the mere use of a pump device, presumably by traumatizing blood cells and releasing vasoactive agents, is often enough to decrease vascular reactivity drastically, as illustrated in Fig. 2 and shown also in earlier studies on other tissues (*cf.* Folkow 1952, Rapela and Green 1964). For such reasons it would have been preferable to arrange for a constant flow perfusion of the type used in the experiment shown in Fig. 1 in all the experiments, but in most cases the animals could not deliver the range of arterial pressure changes needed to keep intestinal blood flow constant at the initial peak vasoconstriction. Thus in deteriorated preparations well sustained increases in resistance to sympathetic stimulation with abolished poststimulatory hyperemia, are sometimes seen which closely resemble the normal intestinal responses to vasopressin infusion. Such observations indicate that the secondary *lowering* of vascular resistance in the proposed 'low resistance channels' in connection with 'autoregulatory escape' is less likely to be due to passive-elastic phenomena but requires a good vascular reactivity. It also follows that these 'low resistance channels' can hardly be opened up when vasopressin is given. Whichever the case, it is evident that quite specific and locally restricted vascular reactions must take place whose complexity makes it impossible to determine the exact events with present technique. Microcirculatory studies and further analyses of the distribution of vasoconstrictor fibres to different sections of the intestinal vessels will be of great value in the further analyses of intestinal vascular events.

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References

Folkow, B., A critical study of some methods used in investigations on the blood circulation. *Acta physiol. scand.* 1952. *27.* 118—129.

Folkow, B., D. H. Lewis, O. Lundgren, S. Mellander and I. Wallentin, The effect of graded vasoconstrictor fibre stimulation on the intestinal resistance and capacitance vessels. *Acta physiol. scand.* 1964 a. *61.* 445—457.

Folkow, B., D. H. Lewis, S. Mellander and I. Wallentin, The effect of the sympathetic vasoconstrictor fibres on the distribution of capillary blood flow in the intestine. *Acta physiol. scand.* 1964 b. *61.* 458—466.

Norberg, K. A., Pers. commun. 1965.

Rapela, C. E. and H. D. Green, Autoregulation of canine cerebral blood flow. *Circulat. Res.* 1964. 15. Suppl. I. 205—211.

Spanner, R., Neue Befunde über die Blutwege der Darmwand und ihre funktionelle Bedeutung. *Morph. Jb.* 1932. *69.* 394—454.

Texter, E. Jr., Choi Ching-Chung, Steven L. Merrill, Higino C. Laureta and E. D. Frohlich, Direct effects of vasoactive agents on segmental resistance of the mesenteric and portal circulation. *J. Lab. clin. Med.* 1964. *64.* 624—633.

Tsakiris, A., U. P. Haemmerli and A. Bühlmann, Reduction of portal venous pressure in cirrhotic patients with bleeding from oesophageal varices by administration of a vasopressin derivative phenylalanine lysine vasopressin. *Amer. J. Med.* 1964. *36.* 825—839.

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Intestinal Dipeptidases

Dipeptidase Activity in the Mucosa of the Gastrointestinal Tract of the Adult Human

By

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Abstract

[illegible]

tion about the intestinal dipeptidases

Knowledge of the terminal proteolytic enzymes in the human intestinal mucosa under normal and pathological conditions is scanty Only a few studies concerning the dipeptidases in this tissue have been reported Several years ago Berger and Johnson (1940) demonstrated some dipeptide hydrolyzing activities in the human duodenal mucosa and more recently Messer, Anderson and Townley (1961) showed that some dipeptides were split by the duodenal mucosa from normal and coeliac children In addition results have also been published suggesting that gluten induced enteropathy may be due to lack of some peptidase(s) in the intestinal mucosa (Frazer 1962, Messer Anderson and Hubbard 1964) It was therefore of interest to investigate some dipeptidase activities in the human intestinal mucosa Previous reports have dealt with intestinal dipeptidases from human fetuses between 11 and 23 weeks of fetal age (Lindberg 1966 The present report describes some properties and the distribution of five dipeptidase activities in the gastrointestinal tract of the adult human

Materials and methods

Dipeptides L-Alanyl L glutamic acid L-alanyl L-proline glycylglycine glycyl L-leucine and glycyl L-valine previously characterized (Josefsson and Lindberg 1965 a Lindberg and Josefsson 1966), were used in the same concentrations as reported earlier Josefsson and Lindberg 1965 a Lindberg and Josefsson 1966)

TABLE I Effect of bivalent metal ions on the dipeptidase activities of the small intestine of adult human Optimum pH

Metal ion added	M μmoles	ΔA_{570}/min/mgN L-Alanyl L-glutamic acid phosphate buffer	L-Alanyl L-proline phosphate buffer	Glycylglycine phosphate buffer	Glycyl L-leucine borate buffer	Glycyl L-valine borate buffer
Co^{2+}	0	11.2	18.6	1.33	68.7	54.9
	10	4.25	18.9	4.64	38.1	47.1
Mn^{2+}	0	11.2	18.6	1.33	68.7	54.9
	10	8.21	19.1	1.28	45.1	45.4
Mg^{2+}	0	11.2	18.6	1.33	68.7	54.9
	10	10.3	18.4	1.21	63.2	48.9
Zn^{2+}	0	11.2	18.6	1.34	68.7	54.9
	10	13.7	18.4	0.55	46.9	28.3

Amino acids The amino acids were products of Mann Research Labs New York For the assays of the dipeptidase activities the amino acids were used in the same concentrations and combinations as already reported (Josefsson and Lindberg 1965 a Lindberg and Josefsson 1965)

Human material Operation specimens from the gastrointestinal tract of 25 adult humans (11 female and 14 male) served as source of the enzyme solution The age of the patients varied between 22 and 80 years (mean age 58 years) The patients were operated for various disorders as stomach ulcer (3) stomach cancer (4) duodenal ulcer (6) ileus post gastroenterostomy (stomach ulcer (1) terminal ileitis (1) ulcerative colitis (1) carcenoid appendicitis (1) and colon cancer (8) The diagnoses were established by clinical and microscopical findings According to the records none of the patients presented a history simulating some other intestinal disease

Preparation of enzyme solution The specimens were cut from the macroscopically normal part of the resected pieces of the gastrointestinal tract and were immediately put in a cold box (4°) The stomach samples were obtained from the fundus part and were about 4 cm² of size Sections of 1—2 cm were obtained from the small and large intestines During the transport to the laboratory the specimen was chilled with ice The enzyme solution was either prepared at once or the specimen was frozen (20° and the enzyme solution prepared the next day In four cases the samples were stored frozen (20°) 4 to 11 days before the enzyme solution was prepared Control experiments performed on mucosa stored frozen revealed that the dipeptidase activities

[illegible] mucosa was carefully cleaned [illegible] paration was homogenized in [illegible] in (MSE homogenizer) After [illegible] gation (International model HR 1 27 000 × g 30 min) The clear supernatant was used directly as the enzyme solution after suitable dilution with distilled water

Assay The dipeptidase activities were assayed according to Josefsson and Lindberg (1965 a) [illegible] The pH values of the [illegible] xide as catalyst. [illegible] role of dipeptide

TABLE II Specific activities (units per mg nitrogen) of dipeptidases in the mucosa of the stomach of adult human Optimum pH

Patient	Sex	Age in years	Diagnosis	L-Alanyl-L-glutamic acid	L-Alanyl-L-proline	Glycyl-glycine	Glycyl-L-leucine	Glycyl-L-valine
A L H [1]	♀	*36*	*Duodenal ulcer*	*10 2*	*0 18*	*3 74*	*31 1*	*25 5*
G P	♂	38	Duodenal ulcer	7 14	0 60	4 41	64 6	35 9
E R	♂	50	Duodenal ulcer	11 1	0 62	4 25	88 1	65 4
A S	♂	64	Duodenal ulcer	12 2	0 64	3 84	64 1	47 5
J H	♂	29	Duodenal ulcer	5 97	0 57	1 75	48 8	30 4
E M	♀	53	Duodenal ulcer	17 8	1 24	6 92	106 5	76 0
A E	♀	49	Stomach ulcer	9 12	0 92	4 92	54 4	45 9
G L [1]	♀	60	Stomach ulcer	34 1	2 19	8 87	153 8	150 3
A J	♀	62	Stomach cancer	18 9	1 43	3 20	95 7	49 3
S S	♂	74	Stomach cancer	9 50	1 08	2 96	39 4	45 2
Mean value				13 6	0 95	4 49	74 7	57 1

[1] *Mucosa from the proximal duodenum was also investigated (Table III)*

Results

Influence of pH and metal ions

Enzyme preparations from the small intestinal mucosa were used in these experiments The pH of the digest mixtures was varied between 5 5 and 9 2 by using 0 15 M phos-
ph [illegible]
act [illegible]
da [illegible]
glycyl-L-valine dipeptidase 7 6

The effect of bivalent metal ions (Co², Mn², Mg² and Zn² ions) upon the various activities were studied at their respective pH-optimum The results obtained are presented in Table I, where the activity is expressed as the decrease in absorption per min and mg nitrogen

It was found that the activity for L-alanyl L-glutamic acid was inhibited about 60 % by Co²⁺ ions and 25 % by Mn² ions while the other two metal ions had no definite influence upon the reaction

TABLE III Specific activities (units per mg nitrogen) of dipeptidases in the small intestinal mucosa of adult human Optimum pH

Patient	Sex	Age in years	Diagnosis	Part of intestine	L-Alanyl L-glutamic acid	L-Alanyl L-proline	Glycyl glycine	Glycyl L-leucine	Glycyl L-valine
G L.[1]	♀	60	Stomach ulcer	First cm of duodenum	3 22	1 08	0	25 7	23 7
A L.H [1]	♀	36	Duodenal ulcer	5 cm from pylorus	8 38	1 66	5 39	31 9	31 3
K P	♀	80	Stomach ulcer	Proximal duodenum	13 4	2 63	5 40	70 6	37 8
P S	♂	76	Stomach cancer	Proximal duodenum	20 9	0 79	7 71	118 7	66 5
E.A	♀	56	Stomach cancer	Proximal jejunum	30 1		10 1	298 8	125 2
E.A	♀	77	Colon cancer	Proximal jejunum	20 1	3 72	9 88	202 6	111 2
E.A	♀	43	Ileus post gastro-enterostomy (Stomach ulcer)	Middle jejunum	28 7	8 45	6 61	195 5	97 9
T H [2]	♂	49	Ulcerative colitis	Terminal ileum[3]	33 3	6 42	9 70	221 0	200 3
B S [2]	♂	22	Terminal ileitis	Terminal ileum[3]	40 6	10 7	11 4	232 0	211 0
F O [2]	♂	76	Colon cancer	Terminal ileum[3]	51 5	13 8	19 4	300 9	223 5

[1] Mucosa from the stomach was also investigated (Table II)
[2] Mucosa from the large intestine was also investigated (Table IV)
[3] 15 30 and 15 cm respectively above the ileo-coecal valve

L-alanyl L-proline dipeptidase activity showed no influence by any of the four metal ions

The rate of the hydrolysis of glycylglycine was increased threefold by Co^{2+} ions The Zn^{2+} ions reduced the rate of the hydrolysis with about 50 %, while Mn^{2+} and Mg^{2+} ions had no influence

The activity against glycyl L-leucine was inhibited by the Co^{2+} Mn^{2+} and Zn^{2+} ions with about 45 % 35 % and 30 % respectively The Mg^{2+} ions were without influence

The glycyl L-valine dipeptidase activity was slightly inhibited by the Co^{2+} and Mn^{2+} ions, while the Zn^{2+} ions reduced the activity about 50 % The Mg^{2+} ions had also in this system no influence

TABLE IV Specific activities (units per mg nitrogen) of dipeptidases in the large intestinal mucosa of adult human Optimum pH

Patient	Sex	Age in years	Diagnosis	Part of intestine	L-Alanyl L-glutamic acid	L-Alanyl L-proline	Glycyl-glycine	Glycyl L-leucine	Glycyl L-valine
F H	♂	49	Ulcerative colitis	Colon[2] ascendens	6 78	0 79	3 11	42 3	34 1
B S [1]	♂	22	Terminal ileitis	Colon ascendens	11 6	2 03	3 00	73 4	56 2
F O [1]	♂	76	Colon cancer	Colon ascendens	13 2	1 85	4 47	62 0	48 3
K G	♀	41	Carcinoid appendicitis	Colon ascendens	11 3	0 89	2 49	66 5	51 6
A E	♂	65	Colon cancer	Colon transversum	9 38	1 85	3 50	49 2	38 6
G N	♂	66	Colon cancer	Colon transversum	16 2	2 13	5 25	90 2	56 7
K L.	♀	68	Colon cancer	Colon transversum	10 1	1 75	3 48	64 6	56 1
A P	♂	73	Colon cancer	Colon descendens	6 05	0 90	3 94	41 2	41 9
B N	♂	75	Colon cancer	Sigmoideum	4 47	1 03	3 04	37 5	32 9
B A	♂	64	Colon cancer	Sigmoideum	6 12	0 67	1 91	48 2	28 7
Mean value					9 52	1 39	3 42	57 5	44 5

[1] Mucosa from the terminal ileum was also investigated (Table III)
[2] The mucosa was macroscopically abnormal

Dipeptidase activity in the mucosa of the stomach small intestine and large intestine

The dipeptidase activities were assayed under optimal pH conditions and in the abscence of metal ions The activities in the mucosa of the stomach expressed as units per mg nitrogen present in the enzyme solution were studied in 10 patients and the results are presented in Table II The dipeptidase activities were as a whole low in comparison with those in the jejunum and ileum see below The small intestinal mucosa was investigated in 10 patients (Table III) The activities were low in the mucosa from the proximal duodenum while the mucosa from the jejunum and terminal ileum constantly contained large amount of dipeptidase activities In Table IV finally the activities obtained from 10 samples of the mucosa of the large intestine are given The activities were found to be on about the same level in the various parts of the large intestine and their amount was comparable to those observed in the stomach

Discussion

When comparing the properties of the various activities observed in the adult mucosa with those in fetal mucosa (Lindberg 1966) some smaller differences of the influence of pH and bivalent metal ions were found Thus the pH optima for the hydrolysis of L-alanyl L-glutamic acid, glycyl L-leucine and glycyl L-valine were slightly lower and the pH-optimum for the hydrolysis of glycylglycine slightly higher than observed in the fetal intestine The inhibiting effect of the Mn^{2} ions on the activity against L-alanyl L-glutamic acid and of the Zn^{2} ions on the activity against glycylglycine in the adult mucosa was not observed in the fetal mucosa The rate of the hydrolysis of glycyl L-valine was decreased by the Co^{2} ions and not influenced by the Mg^{2} ions in the adult mucosa while these metal ions slightly increased this activity in the fetus

These differences may suggest a change of the enzyme properties during the development from the fetal to the adult life but certain conclusion must await further studies

It is known from the previous investigations (Josefsson and Lindberg 1965 b Lindberg 1966 Lindberg and Josefsson 1966) that there is a rather great variation of the dipeptidase activities from one intestine to another In the present study although the various samples of the small intestine were taken from different individuals uniform values were obtained from the various regions of the intestine It may therefore be allowed to compare the distribution of the dipeptidase activities presently observed with the earlier findings in the human fetus and in other species It is then found that the distribution of the various dipeptidase activities in the proximal part of the adult small intestine is more comparable with the one observed in the adult pig (Josefsson and Lindberg 1965 b) than with the human fetus (Lindberg 1966) The activities in the adult duodenum were low while relatively high activities were found in the fetal duodenum However proportionally larger pieces of the intestine were investigated in the fetuses why the results from this region of the small intestine are not quite comparable In the adults the maximal activities were found in the terminal ileum while the maxima were localized within the proximal third of the intestine in the fetuses and in the middle part of the intestine in the pig and the rat (Lindberg and Josefsson 1966 Robinson and Shaw 1960) When considering the amount of the various specific activities it was found that the activities in the adult and fetal jejunum were of about the same magnitude In the terminal ileum on the other hand the activities were higher in the adults than in the fetuses The relatively low activities found in the adult stomach and large intestine are comparable with the amount observed in the adult rat (Lindberg and Josefsson 1966 Robinson 1960) and differ from our observations in the human fetus where the stomach contained a lower and the large intestine a higher amount of activities than in the adult However the mucosa only served as the source of the enzyme solution in the adults whereas in the fetuses the whole wall was used This difference may be responsible for the higher values of the specific activities (units per mg nitrogen present in the enzyme solution) observed in the adults

In recent years it has been shown that dietary protein leaves the small intestine in the form of free amino acids (Dawson and Holdsworth 1962 Levenson Rosen and Upjohn 1959) and that dipeptides are hydrolyzed before or during their transfer through the intestinal wall (Agar Hird and Sidhu 1953 Newey and Smyth 1960 Wiggans and Johnston 1959) Therefore it may be suggested that the distribution of the dipeptidases along the small intestine indicates the capacity of the various parts of the intestine to accomplish the final digestion of the proteins This interpretation of the distribution of

the dipeptidase activities found in the adult human appears to be in conflict with the findings (Borgstrom *et al* 1957, Crane and Neuberger 1960) that the dietary protein is hydrolyzed and absorbed in a rather short time after ingestion and that 80—90 % is absorbed in the upper jejunum The dietary protein constitutes, however, only a minor portion of the protein found in the intestinal lumen (Nasset 1964) The major part, formed by the endogeneous protein, is hydrolyzed and absorbed in the more distal part of the small intestine, where also the dipeptidase activities are found to be highest

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References

AGAR W T E J R HIRD and G S SIDHU, The active absorption of amino acids by the intestine *J Physiol* (Lond) 1953 *121* 255—263

BERGER J and M J JOHNSON The occurence of leucylpeptidase *J biol Chem* 1940 *133* 157—172

BORGSTROM, B, A DAHLQVIST G LUNDH and J SJOVALL Studies of intestinal digestion and absorption in the human *J clin Invest* 1957 *36* 1521—1536

CRANE C W and A NEUBERGER The digestion and absorption of protein by normal man *Biochem J* 1960 *74* 313—323

[illegible]

and characterization of dipeptidase activity in pig intestinal mucosa *Biochim biophys Acta* (Amst) 1965 a *105* 149—161

JOSEFSSON L and T LINDBERG Intestinal Dipeptidases II Distribution of dipeptidase activity in the small intestine of the pig *Biochim biophys Acta* (Amst) 1965 b *105* 162—166

JOSEFSSON L and T LINDBERG Intestinal Dipeptidases III Characterization and determination of dipeptidase activity in adult rat intestinal mucosa *Acta physiol scand* 1966 *66* 410—418

LEVENSON S M M ROSEN and H L UPJOHN Nature and appearance of protein digestion products in upper mesenteric blood *Proc Soc exp Biol* (N Y) 1959 *101* 178—180

activity in the small intestine of human fetus *Clin Sci* 1965 a In press

LINDBERG T Intestinal Dipeptidases Characterization development and distribution of intestinal dipeptidase of the human fetus *Clin Sci* 1966 *30* 505—515

MESSER M CH M ANDERSON and R R W TOWNLEY Peptidase activity of biopsies of the duodenal mucosa of children with and without coeliac disease *Clin chim Acta* 1961 6 768—775

MESSER M CH M ANDERSON and B HUBBARD Studies on the mechanism of destruction of the toxic action of wheat gluten in coeliac disease by crude papain *Gut* 1964 *5* 295—303

NASSET F S *The nutritional significance of endogenous nitrogen secretion in non ruminants* In MUNRO H N (Ed The role of the gastrointestinal tract in protein metabolism Blackwell Scientific Publ Oxford 1964 83—96

NEWEY H and D H SMYTH Intracellular hydrolysis of dipeptides during intestinal absorption *J Physiol* Lond) 1960 *152* 367—380

ROBINSON G B The hydrolysis of dipeptides by rat intestinal extracts *Biochim biophys Acta* (Amst) 1960 *44* 386—387

ROBINSON G B and B SHAW The hydrolysis of dipeptides by different regions of rat small intestine *Biochem J* 1960 *77* 351 356

WIGGANS D S and J M JOHNSTON The absorption of peptides *Biochim biophys Acta* (Amst) 1959 *32* 69—73

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Effect of Respiratory Acidosis on Vasoconstrictor Effects of Directly and Indirectly Acting Sympathomimetic Amines in Cats

By

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Abstract

Bygdeman, S *Effect of respiratory acidosis on vasoconstrictor effects of directly and indirectly acting sympathomimetic amines in cats* Acta physiol scand 1966 *66* 444—447 — The peripheral vasoconstrictor effects of bilateral carotid occlusion were compared with those of the direct [illegible] amine, phenylethylamine and carotid occlusion remained unchanged The results suggest that hypercarbia does not interfere with the action of the endogenously liberated transmitter in the synaptic region but blocks the effect of injected noradrenaline and dopamine on receptors situated outside this region

It has earlier been observed that hypoventilation or respiratory acidosis can strongly reduce or abolish the pressor effect of i v injections or infusions of NA[1], while at the same time the blood pressure response to bilateral occlusion of the common carotid arteries is unchanged or even increased (Dunér and Euler 1959, Bygdeman 1963) The same difference in response pattern during acidosis following hypercarbia has also been found in the perfused cat hindleg between reflex or electric stimulation of the sympathetic vasoconstrictor nerves on one hand and i a injections of NA on the other (Bygdeman 1963) These results thus indicate that the vasoconstrictor effect of injected NA changes independently of that of the endogenously liberated neurotransmitter during a period of acidosis Somewhat similar results have been reported by Manley (1963) in a study on heart refractoriness during respiratory acidosis

To obtain further information concerning this surprising observation the peripheral vasoconstrictor effect of some sympathomimetic amines with different mechanisms of action were tested in the present study before, during and after a period of respiratory acidosis The amines used were noradrenaline, dopamine, tyramine and phenylethylamine

Methods

Cats weighing between 2 3 and 4 0 kg anesthetized with sodium pentobarbital (Nembutal®, Abbott 35 mg/kg i p) were used The following physiological functions were recorded continuously systemic arterial blood pressure vascular resistance in a perfused hindleg arterial

[1] Abbreviations used noradrenaline NA dopamine DA, tyramine TA and phenylethylamine PEA

sure transducer

A measure of the vascular resistance in the hindlimb was obtained with a constant flow

tained constant by a Sigmamotor pump *The perfusion pressure* was recorded distal to the pump via a pressure transducer Changes in perfusion pressure during the course of the experiment

through the "microcatheter sample cell" of a CO_2 infrared analyzer (Beckman Spinco model LB-1)

I v injections were given through a catheter in the right femoral vein, and i.a slow injections over 20 sec to the perfused hindleg were given by an infusion apparatus in the extracorporeal loop proximal to the pump

Results

The average dose of the sympathomimetic amines which increased perfusion pressure by about 50 mm Hg during ventilation with air was NA 0 060 μg / kg DA 2 1 μg kg, TA 3 5 μg / kg and PEA 0 88 μg / kg

Ventilation with 15 % CO_2 caused an increase in the systemic arterial blood pressure and an increase in the resistance of the perfused leg preparation resulting in a mean increase in the perfusion pressure of 60 mm Hg Concomitantly the arterial blood pH decreased from 7 38 to 6 92 and the end tidal CO_2 tension increased from 35 mm Hg to 133 mm Hg

With an increasing degree of acidosis the peripheral vasoconstrictor effect of NA was markedly reduced while the vasoconstrictor responses to i.a injections of TA or PEA were practically unchanged Fig 1 The same difference in response pattern between NA and TA was also observed in an experiment where the time of the i.a infusion was 10 min instead of 20 sec The difference between paired observations was statistically significant P 0 01) On the other hand the effect of DA decreased during the acidotic period to the same extent as that of NA (Fig 2

Discussion

The previously noted difference between the well maintained vasoconstrictor response to occlusion of the common carotid arteries and the decreased effect of injected NA during respiratory acidosis has been confirmed In addition the present results have shown that sympathomimetic amines with indirect action (Fleckenstein 1953, Burn and Rand 1958), such as TA and PEA remain fully active during respiratory acidosis whereas the action of DA which as assumed to act directly like that of NA, is greatly reduced.

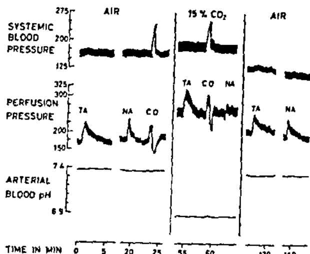


Fig 1 Cat 3 2 kg Left hindleg perfused at a constant blood flow of 9 6 ml/min. Systemic and perfusion pressure responses to i a injections of noradrenaline (NA) and tyramine (TA) and of bilateral carotid occlusion (CO) during ventilation with air and 15 % CO_2 in air TA 6 4 μg/kg, NA 0 02 μg/kg

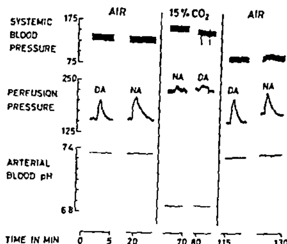


Fig 2 Cat 2 5 kg Left hindleg perfused with a constant blood flow of 8 2 ml/min Perfusion pressure responses to i a injections of NA (0 056 μg/kg) and DA (0 77 μg/kg) during ventilation with air and 15 % CO_2 in air

The decreased effect of injected NA frequently observed during respiratory acidosis (for references see Bygdeman 1963 and Gowdey and Patel 1964) can apparently be due to one or several of the following factors less effective penetration to the site of action, increased rate of destruction decreased sensitivity" of the receptors, or decreased contractility of the vascular smooth muscle

In a recent study of the clearance of labelled NA in the perfused hindleg of the cat (Bygdeman and Stjarne 1965) it was observed that respiratory acidosis only slightly decreased the extraction of the amine from the blood This observation makes it unlikely that the decreased effect of injected NA during acidosis can be attributed to changes in the distribution of the blood flow or decreased penetration of NA into the tissue fluid It is also difficult to understand how a redistribution of the blood flow can influence the fate of injected NA and DA differently from that of TA and PEA

The effect of respiratory acidosis on the rate of enzymatic destruction of injected NA is not known However, Axelrod and Tomchick (1958) have shown that the optimal pH for catechol O methyltransferase *in vitro* is between 7.5 and 8.2 Thus, even if it cannot be excluded that changes in the rate of destruction occur during acidosis, it appears unlikely that this is the main reason for the decreased effect of NA injection It also appears unlikely that the ability of the vascular smooth muscle itself to contract is altered during acidosis since a normal response can be obtained with adequate stimuli

It is now commonly believed that the vasoconstrictor effect of sympathomimetic amines such as NA and DA is exerted by a direct mechanism on the smooth muscles while TA and PEA mainly act by releasing NA from storage sites (Fleckenstein 1953, Burn and Rand 1958) The results obtained in the present study show that the receptors triggering vascular smooth muscles can be normally activated via the sympathetic nerves, but not by exogenous noradrenaline, during a period of respiratory acidosis This finding might be explained, by assuming different locations of the receptors activated by exogenous and endogenous NA Thus, the injected amines DA and NA reach receptors all over the vascular smooth muscle surface by filtration and diffusion into the interstitial space In the case of TA and PEA which are supposed to release the transmitter from intraaxonal stores, this may essentially take place at the "synapse", as in the case of nerve stimulation The discrepancy in the effect between injected NA and endogenously liberated NA during acidosis may thus be explained by assuming that the local conditions for activating the receptor are less altered by acidosis in the "synaptic" region than at other receptor sites The reasons for this difference are still obscure However, it is tempting to speculate on the possibility that the receptors at the "synapse" are better protected against changes in the physico-chemical composition of the extracellular fluid than other parts of the effector cell including its extrasynaptic receptors

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References

AXELROD, J and R TOMCHICK Enzymatic O methylation of epinephrine and other catechols *J biol Chem* 1958 *233* 702—705

Bu [illegible] animals treated with [illegible]

By [illegible]

By [illegible]

D [illegible] and blood flow in the hind part of the cat during infusion of noradrenaline *Acta physiol scand* 19.9 *46* 201—208

FLECKENSTEIN A Konstitution und Wirkung sympathomimetischer Amine Neue Gesichtspunkte nach Versuchen an der normalen und denervierten Nickhaut der Katze *Vaunyn Schmiedeberg s Arch exp Path Pharmak* 1953 *218* 117—119

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Acta physiol. scand. 1966. 66. 448—460

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Entorhinal Activation of Dentate Granule Cells

By

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Abstract

Andersen, P B Holmqvist and P E Voorhoeve *Entorhinal activation of dentate granule cells* Acta physiol scand 1966 66 448—460 — In rabbits, anesthetized with urethane-chloralose, the activation of the granule cells of the dentate area by perforant path fibres from the entorhinal area was studied by intracellular and extracellular recording Intracellular recording showed that the perforant path produced a large EPSP in the granule cells followed after 2—4 msec by a large IPSP lasting some 100 msec Field potential studies indicated that the synapses responsible for the EPSP were located on the middle third of the dendritic tree whereas the IPSP was generated by synapses at or very close to the cell bodies The most likely explanation is that the inhibition is mediated by a recurrent inhibitory pathway in which the collaterals of the granule cell axons excite the basket cells These are inhibitory in nature and send their axons to terminate upon the somata of many granule cells The inhibition of the granule cells produced by the perforant path is resistant to strychnine in doses up to 0 6 mg/kg The efficiency of the perforant path excitatory synapses was greatly increased by raising the rate of stimulation from 1 to 10 a second

The entorhinal area provides the most important afferent inflow to the hippocampus and the dentate area Cajal 1911, Lorente de Nó 1934, Blackstad 1958 These afferent fibres form two distinct pathways One is the alvear path of Lorente de Nó (1934) that distributes itself to the subiculum, the other is the perforant path which terminates in the stratum moleculare of the fields CA1, CA2, CA3 in the hippocampus and in the corresponding layer of the dentate area (Fig 1) In the dentate fascia, the entorhinal afferents, the perforant path fibres make synaptic contact with the dendritic branches at a distance of about 100 μ or more from the soma (Blackstad 1958) (Fig 1B), and have an excitatory effect Andersen and Loyning 1962, Fujita 1962, Gloor, Sperti and Vera 1962) The commissural fibres on the other hand, terminate on the proximal part of the

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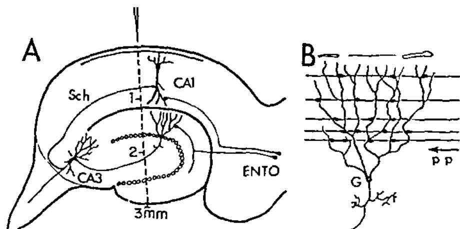


Fig 1 *Diagram over the afferent pathways from the entorhinal area (ENTO) to the hippocampal formation* A The normal track of the microelectrode is indicated by the stippled line which is marked with bars at 1, 2 and 3 mm Sch — Schaffer collateral *B* shows the perforant path (p p) synapsing with the dendrites of the granule cell (G)

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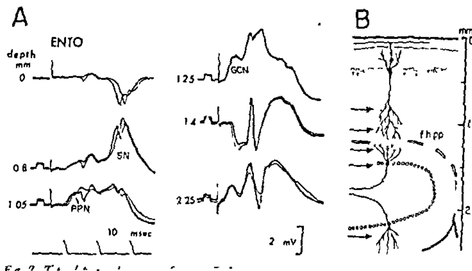
ramifications form around the cell bodies of the granule cells Basket cells in the hippocampal fields CA1 and CA3, and in the cerebellum have been shown to produce large IPSPs in the somata of pyramidal and Purkinje cells respectively (Andersen Eccles and Løyning 1964, Andersen Eccles and Voorhoeve 1964)

The aim of the present investigation has been first to utilize the peculiar synaptic arrangement in the dentate fascia to study with intracellular electrodes the excitatory potency of synapses located at a distance from the cell body Second as a test of the generality of a pattern of somatic location of inhibitory synapses (Andersen Eccles and Løyning 1964 Andersen Eccles and Voorhoeve 1964 Andersen and Eccles 1965) it was of interest to see whether the basket cell terminals on the granule cell somata could produce IPSPs

Methods

Ad

in cats Apart from slightly different depth measurements the characteristics of the potential pattern were the same as in rabbits The hippocampal formation was exposed on both sides by removal of the overlying neocortex and the corpus callosum and the electrodes placed under visual control From the diagram in Fig 1A it is seen that a penetrating electrode will traverse the hippocampal field CA1 for about 1 2 mm before entering the dentate area After an additional 0 4 mm through the dentate molecular layer the electrode will reach the first layer of granule cell bodies called the granular layer After penetrating the hilus of the dentate fascia the electrode will traverse the granular layer for the second time and then the molecular layer because of the curved structure of the dentate fascia



The recordings were made both with surface electrodes and glass micropipettes The surface records were made with 0 5 mm thick platinum wire insulated with polyethylene and cut off squarely, leaving a flat face to rest on the hippocampal surface The insulation prevented

usually 1 sec The signals obtained from the micro-electrode were also fed to a display oscilloscope with a large picture tube having a phosphor with a long afterglow facilitating the study of responses from very short lasting cell impalements

Histological checks were made b

Results

Hippocampal surface potentials following entorhinal stimulation With the recording electrode placed on the surface of the field CA1 of the hippocampus, there was a quite typical potential in response to a stimulus delivered to the entrohinal area or to its efferent projection, the perforant path (Fig 2) A small positive spike of 8 msec latency was followed by a slow wave of about 17 msec latency In order to find out what part of this potential could be generated in the dentate area itself, an electrode was inserted vertically to the alveus of the CA1 and records were taken at various distances from the alveus It was regularly found that only minor changes of the potentials could be detected in the superficial millimeter of the hippocampal cortex At a depth of about 1 0 mm, however, there appeared a negative wave of 4—5 msec latency-PPN, possibly

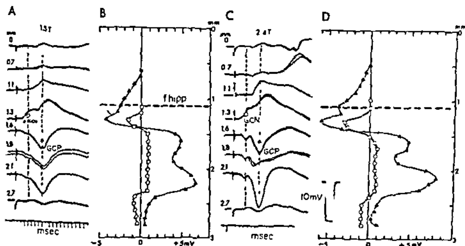


Fig 3 *Laminar analysis of the extracellular field responses of the dentate area in response to entorhinal stimulation* In *A* and *C*, extracellular records taken at 1 5 × threshold and 2 4 × threshold, respectively, as recorded from the depths indicated by the numbers to the left of each record
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related to the depolarization of the terminal dendritic branches of CA1 pyramids by perforant path fibres On further penetration, a new negative wave appeared at about 1 3 mm deep to the hippocampal fissure, having a latency of only about 2 msec (GCN) This wave probably represents the EPSPs that are produced in dentate granule cells by the perforant path volley This early negativity was succeeded by a series of irregular deflections At 1 4 mm, the GCN wave had reversed its polarity, and was followed by a negative spike that probably signalled the discharge of granule cells At 2 25 mm depth, the potential appeared similar to that observed at 1 25 mm, indicating that the electrode recorded from the second layer of granule cells Fig 3 shows the size and polarity of the two components of the potential that can be ascribed to granule cell activity The components are the early negative wave at about 1 3 mm (GCN — open circles), and the positive wave observed at deeper levels (GCP — filled triangles) In the diagram the level of the hippocampal fissure, determined from measurements on histological sections is indicated by the broken line In Fig 3A, the entorhinal stimulus was 1 5 times threshold (T) and in Fig 3C, it was 2 4T The plottings are in Fig 3B and D respectively The GCN wave (open circles) was negative at depths from 1 3 to 1 5 mm, corresponding to the outer two thirds of the molecular layer of the dentate fascia and to the area of termination of entorhinal fibres (Blackstad 1958) In most cases an equivalent negative wave was found at a deeper level In Fig 3B and D, it was at about 2 5 mm depth, corresponding to the lower blade of the molecular layer of the dentate fascia

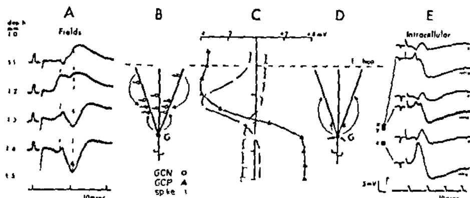


Fig. 4. Extracellular field potentials in relation to the depths at which intracellular penetration took place [illegible]

The peak of the GCN wave corresponds to the location of the perforant path synapses. *D*, Flow of current at the time indicated by the right vertical stippled line in *A* corresponding to the onset of the IPSP. *E*, The lower traces of each pair was obtained by impalement of three cells x, y, z, whose depths are indicated by the filled circles. The upper traces in each pair is the record taken just extracellularly with the same gain and polarity as the intracellular record.

The GCP wave (filled triangles) shows two conspicuous amplitude peaks in Fig. 3B and D, at a depth of 1.6 mm and at 2.2 mm. These depths correspond very closely to the two blades of the granular layer (Fig. 1).

Because of the complex histological arrangement in this area, it is not possible to deduce from extracellular records only, to what extent the deflections recorded in the dentate area are the results of active synaptic depolarizations or hyperpolarizations of parts of the dentate granule cells, and what is due to a passive flow of current. It was, therefore, necessary to correlate the extracellular pattern with intracellular records. However, it proved extremely difficult to obtain satisfactory impalements of the granule cells. This was not unexpected, since the diameter of the granule cell bodies are only 10 to 15 μ, and since they also have an extensive dendritic tree which is very likely to be damaged by the microelectrode approaching the soma. However, it was possible to penetrate 10 cells for a sufficiently long time to allow the determination of the perforant path synaptic activity on the cell.

Since the soma most likely forms the only target for the impaling microelectrode, the depth of the electrode when penetrating cells will give the level of the granule cell bodies as shown in Fig. 4E. All records are taken from the same experiment. In Fig. 4A there are field potentials recorded at the indicated depths below the surface of the alveus, and in C, the amplitudes of the GCN wave (open circles), the GCP wave (filled triangles), and the spike (open squares) are plotted against the recording depth. The level of the hippocampal fissure is indicated by the broken line. The components are measured at the two broken lines in A. The different components demonstrate a typical sequence at increasing depths (Fig. 4C), comparable to that in Fig. 3. The spike was rather small since the stimulus stregnth intentionally was kept relatively weak.

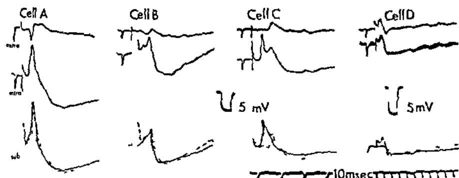


Fig 5 *Intracellular records of dentate granule cells* Upper row Extra and intracellular records from four gran le all
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betwee
A B a
All IPSPs are of long duration whereas the EPSP duration is usually of the order of 5 msec

Since the GCN wave had a short latency and a definite maximum at a depth corresponding to the termination of the afferent perforant path fibres it was considered likely to be the extracellular sign of an EPSP in the granule cell dendrites produced by the entorhinal volley The GCP wave that followed after the spike could either be an active hyperpolarization of the cell, or it could be the source for a current flowing into some distant sink Since the depth recordings showed that the positive wave had two maxima, each corresponding to the two layers of the granular layer, it was anticipated that the positive wave was generated by the granule cells and not by the activity of neurones in the hilus of the dentate fascia

These conclusions were partly confirmed and partly amended by the intracellular records. In Fig 4E, the three filled circles indicate the depths of three granule cells penetrated by the microelectrode and the records obtained are displayed to the right Each pair consists of the intracellular record below with the extracellular record above The large size of the extracellular field potentials in the hippocampal formation makes it imperative always to subtract the extracellular from the intracellular records in order to obtain the potential difference occurring across the impaled membrane It is obvious that the GCN wave is associated with a depolarization of the cell membrane an EPSP This can be more clearly seen in Fig 5 This figure shows the intra and extracellular recordings of four different granule cells In the construction in the lower row, the intracellular potentials are redrawn in broken lines and the extracellular field potentials in dotted lines The fully drawn lines give the subtraction of the two and thus represent the potential changes across the membrane

The intracellular recordings showed an initial depolarizing wave corrresponding to the extracellular GCN wave (Fig 5A B C) Since this depolarizing wave increased gradually with augmenting stimulus strengths and was sometimes associated with granule cell discharges it is an excitatory postsynaptic potential (EPSP) The failure to observe a regular association with impulse generation is no doubt due to the grave depolarization of the small cell by the impalement

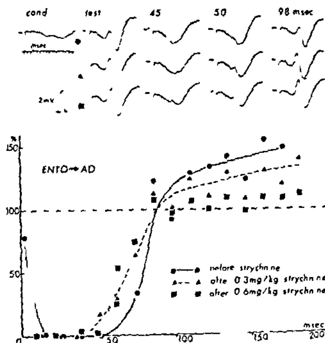


Fig 6 *Inhibition of dentate granule cells* Extracellular records obtained from the granular layer of the dentate area in response to paired entorhinal stimulation. The conditioning shock was [illegible] shows the [illegible] inhibition can be seen, only a reduction in the post-inhibitory facilitatory period

Following the EPSP, there was a large and longlasting hyperpolarization of the neurone This was regularly seen in cells without previous cell discharge (Fig 4, 5), and was associated with cessation of injury or spontaneous discharges and reversed its polarity on chloride diffusion into the cell, it is, therefore, an inhibitory postsynaptic potential, IPSP Correlation with an extracellular potential was possible when the stimulation was weak so that the EPSP was reduced and the onset of the IPSP detectable Under these conditions, it was observed that the extracellular positivity was associated with the initial part of the IPSP (Fig 5B) The duration of the IPSP was around 100 msec The exact amplitude of the IPSP has not much meaning in view of the depolarization due to the damaged cell membrane They were of the order of 5—15 mV

With the information from the intracellular recordings, it is possible to utilize the size of the positive wave at the granular layer as a measure of the intensity of the outward current and thus to localize the focus of the inhibitory synapses It is evident from the depth records in Fig 3 and 4 that the positivity was related to the cell bodies or somata and not to the dendritic tree In the initial phase of the IPSP current is flowing as indicated in Fig 4D

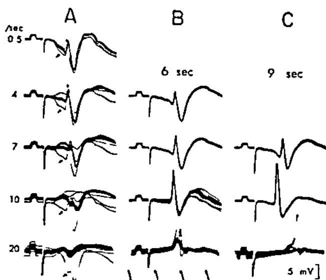


Fig 7 *Frequency* [illegible]

Therefore, the combined use of intracellular and extracellular recordings at various depths leads to the conclusion that the entorhinal afferent volley induces an EPSP in the dentate granule cell dendrites having a maximum at about one third of the distance from the cell bodies to the dendritic terminal branches at the hippocampal fissure This EPSP is followed by a longlasting IPSP which probably is generated at the soma itself

The inhibitory effect of the granule cell IPSPs can be demonstrated by the use of the double shock technique varying the interval between the first conditioning shock and the following test stimulus (Fig 6) The recording electrode was located at a depth of 1 8 mm just deep to the upper blade of the granular layer and the spike signalled the simultaneous discharge of a number of granule cells A preceding conditioning shock (cond), too weak to produce any appreciable occlusion completely depressed all signs of spike discharges in the test response for about 50 msec (upper row and filled circles) Full resumption of the excitability was not attained until after about 90 msec corresponding to the duration of the IPSPs recorded intracellularly Stronger stimulation gave an inhibition lasting for 150 msec Since the inhibition of the granule cell discharge was not dependent of a previous discharge of the same cells the effect was due to a true inhibitory process

Postsynaptic inhibition in the spinal cord is reduced by the administration of strychnine A dose of about 0 2 mg / kg body weight virtually abolishes both the direct and the recurrent inhibition in motoneurones (Bradley Easton and Eccles 1953 Eccles

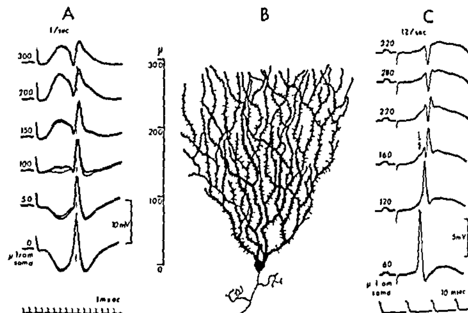


Fig 8 *Propagation of spikes along the dendritic tree of dentate granule cells* Row 1 shows extracellular records taken at the indicated distances from the soma by an electrode that was withdrawn along the dendritic tree The stippled line is drawn through the peak of the spike as seen at the soma layer The increased latency of the peak negativity indicates somatofugal conduction *B* Diagrammatic dentate granule cell drawn to scale to facilitate comparison with the records in *A* and *C* *C* Records take during frequency potentiation (*10 sec*) through the perforant path by a microelectrode being withdrawn from the soma The increasing diphasicity with increasing distances from the soma is indicative of a somatofugal conduction of the spike

Tatt and Koketsu 1954) However several reports indicate that postsynaptic inhibition in various locations in higher levels of the central nervous system is resistant to strychnine (Green, Mancia and Baumgarten 1962 Andersen *et al* 1963 Crawford *et al* 1964) For this reason it was of interest to test whether the postsynaptic inhibition in the dentate granule cells was strychnine resistant or not In Fig 6, the second row is similar to the records in the upper row but taken 2 minutes after an intravenous injection of 0 3 mg strychnine hydrochloride per kg body weight The records of the lower row were taken 4 minutes after an additional dose of 0 3 mg strychnine per kg The graph below is a plotting of the size of the test spike against the conditioning test interval The strychnine administration does not alter the degree or duration of the inhibition but abolished the late facilitation seen at intervals beyond 100 msec Thus the postsynaptic granule cell inhibition is resistant to strychnine in doses up to 0 6 mg per kg

The inhibitory curves and the intracellularly recorded IPSPs both indicate a powerful inhibitory process lasting for about 100—150 msec, hence it was anticipated that stimulation at a frequency of 7 / sec or more would reduce the probability of discharge of the dentate granule cells This was found to be the case In Fig 7A, each assemblage consists of 5—20 superimposed records, the arrows pointing at the response to the first stimulus of the tetanus On increasing the stimulus frequency from 0 5 / sec to 4 / sec, a reduction of about 30 per cent appeared in the height of the population spike

At a rate of 7 / sec, the reduction in spike amplitude was much more marked, and at a stimulus frequency of 20 / sec, the spike was abolished completely This description relates to short lasting tetani (0 5—1 sec) If, however, the tetanic stimulation was maintained for several seconds, the depression was slowly replaced by a facilitatory process, resulting in the reappearance and steady growth of the spike, usually to a larger amplitude than that observed at about 0 5 / sec stimulation (Fig 7B, C, 10 / sec) Further, simultaneous with the increased amplitude of the spike, its latency showed a definite reduction (Fig 7B) This facilitatory process by tetanic stimulation will be called frequency potentiation At higher stimulus rates, the potentiation was soon replaced by an extinction of the cell discharges

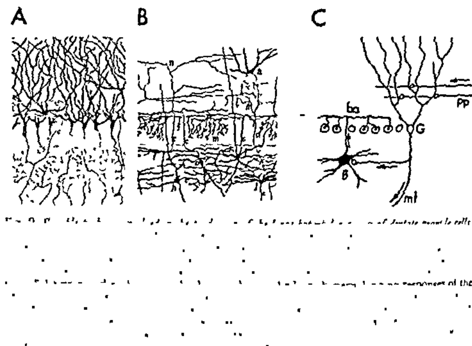
The process of frequency potentiation is slow, requiring several seconds of tetanic stimulation to make itself manifest Conversely, after the cessation of the tetanus a state of increased excitability of the granule cells is observed for several seconds sometimes for as long as half a minute

When the excitability was high, a large number of granule cells discharged virtually simultaneously, and it was then possible to test whether the spike is conducted along the dendritic tree In Fig 8A, the recording electrode was placed at various depths of the molecular layer The response to stimulation at a rate of 1 / sec showed a spike, preceded and followed by a positive wave The electrode was withdrawn in steps indicated by the figures to the left of each record During the withdrawal, the excitability was stable as judged from the appearance of the surface record Initially, the spike was purely negative, corresponding to the thick branches of the dendritic tree Upon further withdrawal, the spike became increasingly diphasic with an initial positivity, until it appeared purely positive at a distance of 300 μ from the soma, corresponding to the distal portions of the dendritic tree The increasing latency of the negative part of the spike during the withdrawal indicates the conduction of the spike along the branches of the dendritic tree until eventually the conduction was blocked about 300 μ from the soma which is very close to the termination of the dendritic branches A comparison of the records taken at 0 and 150 μ from the soma gives a difference in the peak latencies of about 0 3 msec, indicating a mean spike conduction velocity of about 0 5 m/sec

In Fig 8C is shown a similar experiment that was performed in the state of frequency potentiation with a slowly changing excitability The same qualitative changes of the spike configuration occurred at various depths In this case the latency shifts taking place during frequency potentiation prevents the estimation of a conduction velocity

Discussion

Site of the perforant path activation of the dentate granule cells The responses to entorhinal stimulation obtained from an electrode penetrating the dentate fascia showed an early negative wave with a maximum among the granule cell dendrites This component corresponded closely in time to the intracellularly recorded EPSP and is most likely the extracellular sign of this potential The amplitude of the early negative wave can, therefore, be taken as a sign of the intensity of the current flowing inwards through the activated excitatory synapses of the perforant path fibres (Fig 4B) The plotting of the size of the negative wave against depth showed that it had its maximum among the dendrites, some 100 μ from the granule cell somata in the granular layer This localization is in good accord with the histological findings (Blackstad 1958) of degeneration of



[illegible] of dentate granule cells [illegible] responses of the [illegible]

perforant path fibres in the outer two thirds of the molecular layer, with the heaviest concentration of the degeneration in the part bordering the inner third In rabbit, this would give the most massive synaptic excitation on the dendrites at about 100—150 μ from the granule cell somata

Location of inhibitory synapses on the granule cells The extracellular counterpart of the initial part of the granule cell IPSP was the positive wave that follows the spike discharges of the granule cells Even if the positive wave was curtailed by a slow negative wave, it can still be used as an indicator of the intensity of the current flowing outward across the cell membrane due to the activity of the inhibitory synapses (Fig 4D, E) The tracking shows that this current had a maximum at the level of the cell bodies, hence it is concluded that the inhibitory synapses are located at, or very close to the somata of the granule cells

In this respect the inhibitory synapses on the dentate granule cells are similar to those on CA3 and CA1 hippocampal pyramidal cells (Andersen Eccles and Løyning 1964) and on cerebellar Purkinje cells (Andersen Eccles and Voorhoeve 1964), and on neocortical pyramids (Kubota *et al* 1965)

Undoubtedly a part of the positivity at the granular layer is due to this layer acting as a source for the current flowing into the activated excitatory synapse on the dendritic branches about 100 μ from the soma However, this can only apply for the initial part of the granular layer positivity since this wave always outlasted the intracellularly recorded EPSP Furthermore, with the use of weak stimulation the EPSP was small, but both the IPSP and the extracellular positive wave were still present

Evidence for an inhibitory interneurone producing the dentate granule cell IPSPs The wide distribution of the IPSPs, to all granule cells successfully impaled, even when the stimulus was so weak that only a limited number of granule cells were fired, makes it likely that the inhibitory potentials are produced by some mediating cell having wide axonal ramifications This assumption is strengthened by the observation that the EPSP always had a shorter latency than the IPSP recorded from the same cell The quality of the intracellular records do not allow exact measurements to be made of this latency difference, but a range of 2—4 msec was observed

Possible histological substrate for the inhibition of granule cells Having in mind the physiological findings of a wide IPSP distribution and a somatic location of the inhibition, there is hardly more than one histological candidate for the postulated inhibitory interneurone the dentate basket cell. This neurone fulfils all the requirements put forward The axon of each basket cell ramifies profusely and forms, with the axons of fellow cells, basket-like networks around, and makes synaptic contacts with the cell bodies of a large number of granule cells, hence their name (Cajal 1911, Lorente de No 1934) Fig 9A and B is taken from Cajal (1911), showing the granule cells, their axons (Fig 9A) with profusely branching axon collaterals and the basket cells (Fig 9B i, o) with their terminations (Fig 9B, m)

The diagram in Fig 9C explains the hypothesis of entorhinal activation of the dentate area arrived at in the present investigation, to be compared with the histological information in Fig 9A and B The perforant path fibres (pp) terminate in excitatory synapses on the granule cell dendrites, at some distance from the soma, where they evoke EPSPs In some granule cells, the EPSP will be large enough to discharge the cell The impulse travels along the axon the mossy fibre (mf) and also along its numerous axon collaterals, ramifying beneath the graular layer These collaterals are assumed to to excite the cells in the subgranular layer (B), where the majority of neurones are basket cells (Cajal 1911) By way of their terminals (ba) on the granule cell bodies, the basket cells will influence a large number of granule cells The basket cell synapses on the granule somata are proposed to be inhibitory, and responsible for both the large IPSP observed in the granule cells and for the positive wave recorded extracellularly from the granular layer

It is pertinent to mention that the synapses in the cell body of the dentate granule cells are of the type 2 (Blackstad and Dahl 1962), thus being similar to inhibitory synapses identified in the hippocampus (Andersen Eccles and Loyning 1964, Blackstad and Flood 1963 Hamlyn 1963) and on cerebellar Purkinje cells (Andersen Eccles and Voorhoeve 1963 1964, Palay *et al* 1962)

Spike generation and propagation of the granular cells The homogeneity of the dentate fascia with the vast majority of the cells being similar in shape and parallel to each other with the somata at the same depth, allows some conclusions to be drawn regarding the propagation of the spike recorded at various depths The pure extracellular negativity of the spike in the granular layer and the first 100 μ of the molecular layer suggests that the spike is generated either in the soma or more likely in the proximal parts of the dendrites bordering the synaptically depolarized part of the cell The progressive diphasicity and increasing peak latency of the spike with increasing distance from the soma suggest a somatofugal conduction of the spike Conduction of a spike with the same slow velocity has been observed along the apical dendrites of hippocampal pyramidal cells (Cragg and Hamlyn 1955 Andersen 1960 Andersen and Jansen 1961, Fujita and Sakata 1962 Andersen Holmqvist and Voorhoeve 1966)

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References

ANDERSEN P, Interhippocampal Impulses II Apical dendritic activation of CA1 neurons *Acta physiol scand* 1960 *48* 178—208

ANDERSEN P and J C ECCLES Localization of inhibitory synapses on somata of hippocampal [illegible]
in the brain *Nature* (Lond) 1963 *200* 843—845

ANDERSEN P J C ECCLES and P E VOORHOEVE Postsynaptic inhibition of cerebellar Purkinje cells *J Neurophysiol* 1964 *27* 1138—1153

ANDERSEN P B HOLMQVIST and P E VOORHOEVE Excitatory synapses on hippocampal apical dendrites activated by entorhinal stimulation *Acta physiol scand* 1966 *66* 461—472

ANDERSEN P and J JANSEN JR The local cortical response in the hippocampus of rabbit *Arch ital Biol* 1961 *99* 349—368

ANDERSEN P and Y LØYNING Interaction of various afferents on CA1 neurons and dentate granule cells *Coll int CNRS* No 107 Paris 1962 23—42

BLACKSTAD T On the termination of some afferents to the hippocampus and fascia dentata *Acta anat* (Basel) 1958 *35* 202—214

[illegible] of primary or direct inhibition [illegible] Maloine 1911 2

CRAGG B G and L H HAMLYN Action potentials of the pyramidal neurones of the hippocampus of the rabbit *J Physiol* (Lond) 1955 *129* 608—627

CRAGG B G and L H HAMLYN Some commissural and septal connexions of the hippocampus in the rabbit A combined histological and electrical study *J Physiol* (Lond) 1957 *135* 460—485

CRAWFORD J M D R CURTIS P E VOORHOEVE and V J WILSON Strychnine and cortical inhibition *Nature* (Lond) 1963 *200* 845—846

ECCLES J C P FATT and K KOKETSU Cholinergic and inhibitory synapses in a pathway from motor axon collaterals to motoneurones *J Physiol* (Lond) 1954 *126* 524—562

FUJITA Y Synaptic activation of dentate granule cells and its effect upon pyramidal cells in rabbit *Coll int CNRS* No 107 Paris 1962 47—68

FUJITA Y and H SAKATA Electrophysiological properties of CA1 and CA2 apical dendrites of rabbit hippocampus *J Neurophysiol* 1962 *25* 209—222

GLOOR P L SPERTI and C VERA An analysis of hippocampal evoked responses and seizure discharges with extracellular microelectrode and DC-recordings *Coll int CNRS* No 107 Paris 1962 147—170

GREEN J D M MANCIA and R VON BAUMGARTEN Recurrent inhibition in the olfactory bulb I Effects of antidromic stimulation of the lateral olfactory tract *J Neurophysiol* 1962 *25* 467—488

HAMLYN L H An electron microscope study of pyramidal neurons in the Ammon's horn of the rabbit *J Anat* (Lond) 1963 *97* 189—201

LORENTE DE NÓ R Studies on the structure of the cerebral cortex II Continuation of the study of the Ammonic system *J Psychol Neurol* (Lpz) 1934 *46* 113—177

Acta physiol. scand. 1966. 66. 461—472

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Excitatory Synapses on Hippocampal Apical Dendrites Activated by Entorhinal Stimulation

By

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Abstract

Andersen, P, B Holmqvist and P E Voorhoeve *Excitatory synapses on hippocampal apical dendrites activated by entorhinal stimulation* Acta physiol scand 1966 66 461—472 — In rabbits anesthetized with urethane-chloralose the responses produced in the field CA1 by stimulation of the entor[illegible]
path[illegible]
cells [illegible]
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always secondary to dentate granule cell discharges it disappeared after a section severing the Schaffer collaterals at the CA2/CA1 border it showed longer latency the longer the recording ele[illegible]

in that part of the dendritic membrane which borders the territory of the Schaffer synapses The spike was conducted towards the soma with a speed of about 0 4 m sec Intracellular recording from cat CA1 pyramids gave as a rule no sign of soma depolarization in spite of the appearance of synaptically initiated action potentials The results indicate that synapses located to the dendrites can initiate a local spike that may be conducted towards the soma with a low conduction velocity and probably with a low safety factor

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The entorhinal area is regarded as the main source of afferent fibres to the hippocampal formation (Cajal 1911 Lorente de No 1934) Still very little is known about the manner in which the afferent fibres from this region exert their effect on the hippocampus and dentate area The aim of the present investigation has been to delineate the

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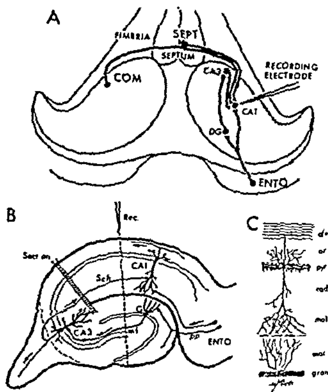


Fig 1 *Diagrammatic representat...* as viewed fror ... of several pat ... *B* Diagram ... CA3 of the t ... drawn in eacl ... (ENTO) forr ... and partly on ... mossy fibres (... course in the fimbria and has each a large branch the Schaffer collateral (Sch) which terminates on the CA1 apical dendrite The section severs all Schaffer collaterals *C* Diagram of a CA1 pyramidal cell above and a dentate granule cell below with the laminar arrangement of the hippocampal formation alv — alveus or — stratum oriens, pyr — stratum pyramidale rad — stratum radiatum mol — stratum moleculare, gran — granular layer

afferent routes by which the entorhinal area activates field CA1 of the hippocampus, and to establish the manner in which the synaptic excitation of the pyramidal cells in this area takes place It was found necessary to relate the events observed in the field CA1 with those occurring simultaneously in the dentate area (Andersen, Holmqvist and Voorhoeve 1966)

The afferent fibres to the hippocampal formation from the entorhinal area form a bundle of fibres, called the perforant path by Lorente de Nó (1934) because they penetrate the subicular, presubicular and parasubicular cortices on their way towards their destination. Referring to the diagram in Fig 1B, it is seen that the perforant path fibres

(pp) end in two areas Some fibres, after they have penetrated the subiculum and neigh-

1958, Hamlyn 1963) The rest of the perforant path fibres penetrate the obliterated hippocampal fissure to end in the molecular layer of the dentate fascia Here they make synaptic contacts with the granule cell dendrites in the outer two thirds of the layer, predominantly in the middle third (Blackstad 1958, Cajal 1911, Lorente de Nó 1934) The axons of the granule cells the mossy fibres (mf), form a dense bundle and make synaptic contact with the proximal portions of the apical dendrites of the CA3 pyramids These cells in turn send their axons out into the fimbria However, impulses in these axons will also travel along their myelinated Schaffer collaterals (Sch) which turn around to penetrate the pyramidal layer and course parallel to the ventricular surface towards and into the field CA1 These collaterals make synaptic contact with the apical dendrites of the CA1 pyramids in a restricted, band like layer, in the rabbit located about 0 3—0 4 mm from the layer of the cell bodies

Thus the enthorinal area has two main routes by which it can influence the CA1 pyramidal cells One is a monosynaptic by way of the perforant fibres ending on the terminal branches of the apical dendrites the other is a trisynaptic pathway involving dentate granule cells and CA3 pyramidal cells The last pathway terminates with synapses on the CA1 apical dendrites closer to the soma than the first pathway, but still at a considerable distance from the pyramidal cell bodies This histological arrangement invites a study of the effect which a peripherally situated population of synapses may have on the membrane potential of the cell body The methods have been described in the previous paper (Andersen Holmqvist and Voorhoeve 1966) Some additional procedures will be described in the text

Results

CA1 potentials elicited by an entorhinal volley In response to stimulation of the entorhinal area or the perforant path fibres a microelectrode that just touches the surface of the alveus over CA1 did only record a potential of small amplitude If however, the electrode was moved into the CA1 cortex vertically to the surface and parallel to the apical dendritic shafts of the pyramidal cells, the electrode soon detected potentials of typical form and of a considerable size (Fig 2A) Three major potentials could be distinguished each one having an optimal size at a specific level These potentials are (i) the *spike* (indicated by a cross in Fig 2A) (ii) the early *PPN wave* (asterisk) and (iii) the later *SN wave* (open squares The reasons for this labelling are given below

The most prominent component at depths from 0 2 to 0 4 mm from the surface was the spike It had a maximum at 0 3 to 0 35 mm which corresponds to the pyramidal cell layer At 0 4 mm it was purely negative and had a duration of about 2 msec More superficially it rapidly diminished and became diphasic with an initial positivity, suggesting its propagation towards the ventricular surface In Fig 2B the amplitude of the various components of the CA1 response to entorhinal stimulation is plotted against the depth of the recording electrode The spike (crosses) had a considerable amplitude from 0 4 down to depths of about 0 6 mm corresponding to the cell bodies and the shafts of the apical dendrites — the *stratum radiatum* — and suggesting that the apical dendritic membrane contributes to the generation of the spike potential

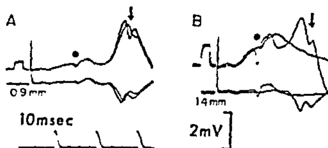


Fig 4 *The dependence of CA1 activity on granular cell activity* *A* Records taken from 0.9 mm depth showing a small deflection (filled circle) as a sign of the distant granule cell activity followed by the SN wave with a superimposed positive spike (arrow) *B* Records taken from a depth of 1 4 mm showing that the all-or nothing appearance of the late activity corresponding to the SN wave (arrow) depends upon whether the granule cells discharge or not. Similar responses were obtained from the surface (lower trace)

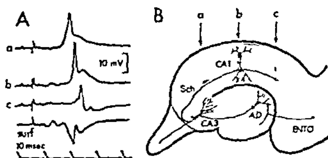


Fig 5 *Direction of the pathway involved in activation of CA1 pyramids* *A* Records taken at a depth of 0 6 mm (a b c) in response to a single entorhinal shock as recorded from the tracks a b, and c (*B*) The lower record in *A* is the surface record taken in conjunction with the *b* record The increasing latency of the SN wave with its superimposed spike when recording from the points *a* to *c* indicates that the activating volley proceeds in the same direction as the Schaffer collaterals (arrow Thus the pathway involved in this activation must be the one indicated in *B*

is mediated by way of the granule cells and CA3 pyramids the SN wave should always be secondary to granule cell discharge In Fig 4 the strength of the entorhinal stimulus was kept just above the threshold of granule cell discharge At 0 8 mm depth (A) the SN wave carried a positive spike on its top (arrow) In the records taken from a depth of 1 6 mm B the same positive spike (arrow) can be used to identify the SN wave This wave occurs only subsequent to granule cell discharge as indicated by the early positive spike dot Thus this experiment leads to the conclusion that the discharge of dentate granule cells is a prerequisite for the SN wave in CA1

Finally the proposed pathway for CA1 activation from the entorhinal area requires that those CA1 cells which lie close to the CA3 should discharge before those lying closer to the subiculum. In the experiment illustrated in Fig 5 the stimulating electrode was kept fixed in the entorhinal area The recording microelectrode was inserted along three tracks a, b and c in a sagittal plane and 2 mm from each other (Fig 5B) The records obtained from a depth of 0 6 mm in each track are shown in Fig 5A labelled

according to the tracks The latency of the SN wave and of the spike increased the greater the distance from the CA3 The only explanation to be offered for this observation is that the activating volley travels in a caudal direction from the region of CA3 A volley along the Schaffer collaterals propagates in the required direction, opposite to that of the perforant path fibres

In conclusion experiments employing section of the Schaffer collaterals, various strengths of the afferent entorhinal volley and recording in various parts of the CA1, have unequivocally shown that the SN wave recorded at a depth of about 0 8 mm in the field CA1, and the spike associated with it are due to a trisynaptic pathway involving the Schaffer collaterals of the CA3 pyramidal neurones The latter cells are activated by the dentate granule cells which initially are activated by the entorhinal volley along the perforant path fibres

Spike generation and propagation The relation between the SN wave and its associated spike may be seen in Fig 2 In Fig 2 the spike was purely negative at depths from 0.5 to 0 8 mm, suggesting that those parts of the pyramidal cell membranes lying between these two depths acted as a sink only This layer corresponds to the shafts of the apical dendrites At more superficial levels from 0 4 up to 0 1 mm, the spike was diphasic with an initial positivity, indicating that the more superficial parts of the cell the cell body and the basal dendrites initially acted as a source for the sink in the apical dendritic shafts Later, these parts of the neurones became depolarized themselves At depths greater than the layer of the apical dendritic shaft the spike appeared as a positive deflection, indicating that the terminal portion of the dendritic tree never was antidromically invaded by the spike It seems clear therefore that with the entorhinal input, the CA1 apical dendritic shaft is depolarized as the initial event and that a spike is initiated and propagated in the somatopetal direction

However, a better indication of the site of generation of the spike and the speed of its propagation can be derived from measurements of the latency of the spike recorded at various depths below the alveus Due to the trisynaptic pathway the expected latency differences along the extent of the pyramidal neurones are relatively small when compared with the total latency from the stimulus artefact Therefore it proved advantageous for accurate measurement to make use of an expanded sweep There is also another important provision to make in order to get reliable latency data There appears in the hippocampal formation spontaneous excitability fluctuations of varying duration leading to randomly occurring alterations in the latency of the spike These variations are all the more important when as in the present investigation a trisynaptic pathway is used Therefore instead of measuring the latency between the stimulus artefact and the spike the latency difference was measured between the spike recorded by the microelectrode and the spike of the surface record Any spontaneous excitability variation will reflect itself simultaneously in the surface and the deep electrode provided these are placed close to each other

In Fig 6A are records taken at the indicated depths below the ventricular surface in response to an entorhinal stimulus The time marker record shows the part of the sweep which was expanded for the latency measurements These expanded sweeps are seen in B where the upper trace is the depth record and the lower is the surface record The first broken line connects the troughs of the positivities of the surface spike (lower traces and the second line is drawn through the peak of the spike recorded at 0 6 mm depth It is evident that both the onset and the peak of the spike have longer latencies with more superficial electrode locations 0 4 and 0 5 mm) In Fig 6C,

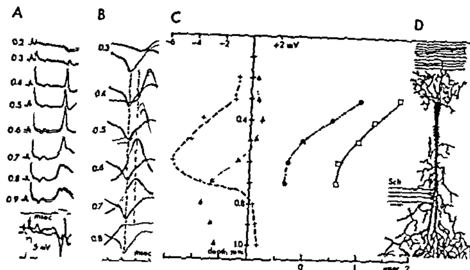


Fig 6 Initiation and propagation of spikes along the CA1 apical dendrites *A* Extracellular records taken at the indicated depths below the ventricular surface in response to entorhinal activation. The square wave superimposed on the time scale indicates the part of the record to be expanded in *B* *B* Expanded sweeps taken at the indicated depths below the surface The upper trace of each pair is the m...

... are plotted against recording depth. To the right in the graph is plotted the initial latency (filled circles and the peak laten...

The increasing laten...

at a velocity of about...

with the records sho...

the size of the negative SN wave and the amplitude and the latency of the spike are plotted against the recording depth. As in Fig 2, the maximum of the SN wave was found at a depth of 0 8 mm corresponding to the transition between the shaft and the thinner branches of the apical dendrites The spike, however, showed its largest size at the somewhat shallower depth of 0 6 mm However, the maximum was a relatively flat one, since the spike was of a considerable amplitude from the level of the cell bodies (0 4 mm) and down to the main branching of the dendrites (0 7 mm) The latency of the spike D) whether measured to its foot (filled circles) or to its peak (open squares) increased from a depth of 0 8 mm to 0 3 mm, giving a conduction velocity ranging from 1 4 to 0 25 m / sec with 0 4 m/sec as the mean value for the propagation along the apical dendritic shaft

The only explanation that can be offered for these observations is that the entorhinal input, through the pathway described is capable of generating a spike in the apical dendrites at, or close to the level of the *stratum lacunosum*, which is where the main branching of the apical dendrite takes place The spike is propagated along the apical dendritic shaft towards the soma with a low speed, having a mean value of about 0 4 m/sec The spike was most likely initiated just superficially to the Schaffer synapses since the latency did not increase until a depth of 0 7 mm

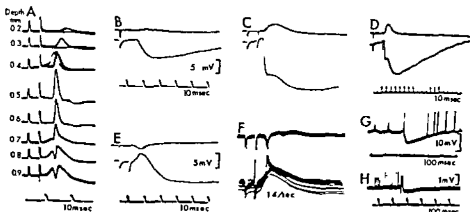


Fig 7 ...

r
l
r
t
c
r
F

examples showing cessation of spontaneous discharges in response to single Schaffer collateral activation

Since the SN wave had a clear relationship to the spike in size and time and since it was confined to a depth just below where the spike started it is likely that the SN wave is the extracellular sign of the EPSP produced by the synapses of the Schaffer collaterals

In order to investigate to what degree this peripherally located Schaffer EPSP makes itself evident in the soma about 400—500 μ away an attempt was made to impale the CA1 pyramidal cells with microelectrodes For some unknown reason this proved very difficult in rabbits However in cats intracellular recording was relatively easy, and a number of CA1 pyramidal cells were penetrated Since the entorhinal input is much more difficult to secure in cats we stimulated the Schaffer collaterals directly in the CA3 region as described by Fujita (1962) and Fujita and Sakata (1962) The pattern of the extracellular potentials were similar to that in rabbits with the SN wave as a prominent component observed at a depth of 0 7—0 8 mm (Fig 7A) Almo...

No sign of a preceding depolarization was seen in the 17 cells successfully impaled not even if the stimulation frequency was raised to produce an increased EPSP due to frequency potentiation of the Schaffer collateral synaptic transmission Only in one cell did a preceding depolarization occur Fig 7E F In this case the penetration occurred at a depth of 0 6 mm about 0 2 mm deep to the pyramidal layer Furthermore the extra-cellular record showed a negative wave as its most important component unlike the positive wave that was found at the level of the cell bodies For these reasons it is possible that the microelectrode impaled the shaft of one of the thick apical dendrites

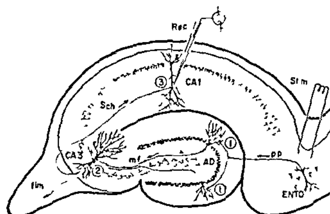


Fig 8 *Proposed pathway for activation of the field CA1 of the hippocampus by entorhinal stimulation* The perforant path (pp) activates the first synapse in the pathway (encircled number 1) through synapses ending on the middle [illegible]
c [illegible]
r [illegible]
b [illegible]
t [illegible]
the perforant path synapses ending on the peripheral part of the CA1 pyramids is not drawn because of its lesser physiological significance

about 0 2—0 3 mm from the location of the Schaffer collateral synapses, or a non-pyramidal cell

These observations make it unlikely that a dendritic EPSP in the CA1 pyramids is capable of making a substantial depolarization of the soma, unless it is situated less than 200 micron from the cell body

Discussion

Pathways for the entorhinal activation of the hippocampal field CA1 From the preported experiments, the following picture emerges regarding the activity initiated by a perforant path volley

The predominant activity in CA1 following entorhinal stimulation is mediated via the pathway outlined in Fig 8 The three synapses involved are indicated by the encircled numbers 1, 2, and 3, according to their sequential activation The conclusion is based upon several observations The most important are the findings that a section of the Schaffer collaterals abolished the CA1 activity and that the latency of the activity in various locations within CA1 indicated that it was evoked by fibres conducting in a caudal direction, directly opposite that of the perforant path fibres

It has been claimed that entorhinal stimulation in cats is followed by a large negative wave confined to the peripheral part of the CA1 dendritic tree This has been interpreted as a summated EPSP generated by the perforant path fibres in the molecular layer (Purpura 1959, Gloor, Vera and Sperti 1963) In view of the histological similarity, it appears unlikely that the physiological organization should deviate much between the rabbit and the cat We are, therefore, of the opinion that the CA1 potentials thought to be initiated by a direct perforant path volley, in fact have been evoked by Schaffer collaterals activated indirectly by the perforant path volley through the discharge of

dentate granule cells (Fig 8) Any claim to a direct effect of the perforant path volley of the peripheral dendritic branches of CA1 must stem from experiments in which the activity mediated through Schaffer collaterals have been eliminated (Fig 1, 5)

The great power of the perforant path fibres activating the CA1 indicates that the entorhinal area is a major source of afferent information to the hippocampal formation This conclusion was drawn already some seventy years ago by Cajal (1893) and later agreed to by Lorente de Nó (1934), both basing their views upon histological investigations

Generation and propagation of spikes along dendrites We are here only concerned with the propagation of spikes of short duration (1—2 msec) There is now good evidence that spikes may be conducted along dendrites of a variety of neurones Lorente de No (1947) as well as Fatt (1957) found that an antidromic volley invades the dendrites of motoneurones There is evidence that spikes may be generated in and propagated along the dendrites of chromatolysed motoneurones (Eccles, Libet and Young 1958) An antidromic spike will invade a considerable portion of the stretch receptor dendrites (Eyzaguirre and Kuffler 1955 Grampp 1966), and an antidromic spike will invade the dendritic tree of cerebellar Purkinje cells (Eccles Llinas and Sasaki 1965)

Turning to the hippocampus, it is clear from measurements of the size, shape and latency of evoked spikes that they may be conducted along the apical dendritic shafts of the pyramidal cells in response to direct stimulation of intracortical afferents (Cragg and Hamlyn 1955), to commissural stimulation (Andersen 1960), to local stimulation (Andersen and Jansen 1961) which most likely represents the antidromic invasion of the pyramidal cells and to the direct stimulation of the Schaffer collaterals (Fujita 1962, Fujita and Sakata 1962) In all these studies the conduction velocity of the dendritic spike has been given as 0 3—0 6 m/sec In the present investigation, the possibility of conduction of a spike along the apical dendritic shaft has been confirmed In addition, it has been shown that the spike is generated peripherally in the dendrite and conducted somatopetally The almost constant amplitude of the spike along the apical dendritic shaft suggests that the safety factor for the spike propagation along this structure may be higher than has hitherto been assumed

The failure to detect any sign of the distally located EPSP by an electrode inserted in the soma suggests that the length constant of the apical dendritic membrane is relatively small Certainly it must be considerably shorter than the distance of the Schaffer collateral synapses from the soma some 400—500 μ Without the possibility of spike propagation along the dendrites these peripherally located synapses would have very little influence over the discharge of the cell However, the synapses of the Schaffer collaterals proved to be very effective in evoking the discharge of pyramidal cells Only the synapses which like the perforant path synapses on the CA1 pyramids are situated on the very finest terminal dendritic branches seen to have little possibility of initiating a spike on their own However by depolarizing the bordering part of the cell membrane they will increase the probability for the neighbouring synapses to bring the cell to discharge

We infer that in addition to the cell body the whole of the apical dendritic shaft is capable of initiating a spike and propagating it towards the soma and eventually along the axon This arrangement gives a far greater importance to the synapses located far from the soma increasing their power in the control of the neuronal discharges than would have been the case if the remote synapses had had to exert their effect by an electrotonical effect only

We wish to thank Professor Sir John Eccles for his valuable help and criticism
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References

ANDERSEN, P, Interhippocampal impulses II. Apical dendritic activation of CA1 neurons. *Acta physiol scand* 1960 *48* 178—208
ANDERSEN, P, J C ECCLES and Y. LØYNING, Recurrent inhibition in the hippocampus with identification of the inhibitory cell and its synapses *Nature* (Lond) 1963 *198* 540—542
ANDERSEN, P, J C ECCLES and Y LØYNING, Location of postsynaptic inhibitory synapses on hippocampal pyramids *J Neurophysiol* 1964 *27*, 592—607
ANDERSEN, P. and J JANSEN JR, The local cortical response in the hippocampus of rabbit *Arch ital Biol* 1961 *99* 349—368
ANDERSEN, P, B HOLMQVIST and P. E. VOORHOEVE, Entorhinal activation of dentate granule cells *Acta physiol scand* 1966 *66*, 448—460.
BLACKSTAD, T W, On the termination of some afferents to the hippocampus and fascia dentata *Acta anat* (Basel) 1958 *35* 202—214
CAJAL, R S R, *Histologie du système nerveux de l'Homme et des Vertébrés* Paris, A Maloine 1911 *2* 993
CRAGG, B G and L H HAMLYN, Action potentials of the pyramidal neurons in the hippocampus of the rabbit *J Physiol* (Lond) 1955 *129* 608—627
ECCLES J C, B LIBET and R R YOUNG The behaviour of chromatolysed motoneurones studied by intracellular recording *J Physiol* (Lond) 1958 *143* 11—40
ECCLES J C, R LLINAS and K SASAKI, Parallel fibre stimulation and the response induced thereby in the Purkinje cells of the cerebellum *Exp Br Res*, 1966 *1* 17—39
EYZAGUIRRE, C and S W KUFFLER Further study of soma dendrites and axon excitation in single neurons *J gen Physiol* 1955 *39* 121—153
FATT, P Electric potentials occurring around a neurone during its antidromic activation *J Neurophysiol* 1957 *20* 27—60
FUJITA Y Synaptic activation of dentate granule cells and its effect upon pyramidal cells in rabbit *Coll int C N R S* No 107 Paris 1962 47—66
FUJITA Y and H SAKATA Electrophysiological properties of CA1 and CA2 apical dendrites of rabbit hippocampus *J Neurophysiol* 1962 *25* 209—222
GLOOR P C L VERA and L SPERTI, Electrophysiological studies of hippocampal neurons I Configuration and laminar analysis of the "resting" potential gradient of the main transient response to perforant path fimbrial and mossy fiber volleys and of "spontaneous" activity *Electroenceph clin Neurophysiol* 1963 *15* 353—378
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LORENTE DE NÓ, R Action potential of the motoneurons of the hypoglossus nucleus *J Cell comp Physiol* 1947 *29* 207 288
PURPURA, D R Nature of electrocortical potentials and synaptic organization in cerebral and cerebellar cortex *Int Rev Neurobiol* 1959 *1* 47—163

Acta physiol. scand. 1966. 66. 473—480

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Potentiation of Acid Sham Feeding Response in Pavlov Pouch Dogs by Histamine and by Mesenteric-Caval Vein Anastomosis

By

LARS OLBE[1]

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Abstract

Olbe, L. *Potentiation of sham feeding response in Pavlov pouch dogs by histamine and by mesenteric-caval anastomosis* Acta physiol scand 1966 66 473—480 — The acid sham feeding responses in Pavlov pouch dogs were almost abolished by resection of the main sources of endogenous gas-trin [illegible]

caval anastomosis

The direct vagal excitation of the gastric HCl glands by physiological means requires the presence of gastrin to produce a significant acid secretion (Olbe 1964 b) A potentiation between the actions of the two stimuli has been demonstrated (Olbe 1964 a) Subthreshold amounts of gastrin are enough to turn the ineffective direct vagal excitation into a marked secretory action (Olbe 1964 a) reinforcing the hypothesis that the cephalic phase of gastric acid secretion is controlled by a neuro-humoral mechanism (Uvnäs 1942) Whether this phenomenon is characteristic to gastrin or is a feature common to other humoral secretagogues was the question initiating the present study

The humoral secretagogues investigated were histamine and an agent released from the post-duodenal intestines into the portal blood — the same agent that has been intensely studied by portacaval transposition during the past few years (Clarke 1960) In addition some pertinent data are given which have been previously presented in a preliminary form (Olbe 1960)

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TABLE I Effect of i v. infusion of subthreshold amounts of histamine (0 078, 0 031, 0 061, 0 048 and 0 389 μg histamine dihydrochloride/kg/min in dogs B, E, M, N and O, cf table II), and anastomosis of the superior mesenteric vein to the inferior caval vein (MCA) or portacaval transposition (PCT) on the mean 3 5 hrs sham feeding response in Pavlov pouch dogs after resection of gastrin releasing regions

Mean acid response to sham feeding

			After resection of gastrin releasing regions					
	Pavlov pouch preparation		Without histamine		With subthreshold amounts of histamine		After MCA or PCT[1]	
Dog	mEq±S E	No of expts	mEq±S E	No of expts	mEq±S E	No of expts	mEq±S E.	No of expts
B	1 05±0 20	4	0 31±0 10	6	6 21±0 89	4	4 21±0 33	6
E	3 26±0 45	5	0 45±0 03	8	2 36±0 56	4	3 22±1 00	4
F	0 76±0 13	4	0 20±0 02	3			2 35±0 39	7
L	0 38±0 07	4	0 04±0 02	5			[1]0 54±0 07	4
M	1 37±0 14	4	0 17±0 03	5	3 45±0 11	4		
N	1 28±0 27	4	0 20±0 04	5	2 19±0 22	4	1 70±0 46	4
O	0 68±0 14	5	0 21±0 04	5	9 14±0 35	4	4 26±0 75	5

Methods

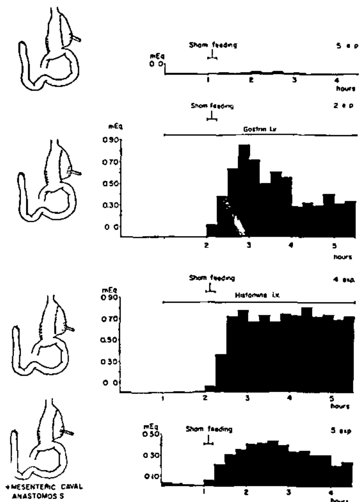
Operative procedures Healthy mongrel dogs were provided with an esophageal cannula (Olbe 1959) and an innervated (Pavlov) fundic pouch (Thomas 1942) or a denervated (Heidenhain) fundic pouch (DeVito and Harkins 1959) Resection of the gastrin releasing regions — the antrum the duodenal bulb and possibly the distal few cm of the HCl-secreting gastric mucosa — was performed after peroperative visualization of the antrum-corpus boundary as previously described (Olbe 1963 Olbe 1964 b) Mesenteric caval anastomosis was produced as an end-to-side shunt between the superior mesenteric vein that was transected just *before joining* the splenic vein and the inferior caval vein Portacaval transposition was performed according ... of the transposition

jejunum

... experiment that started with ... ch ... nst ... ed

3 1/2 hrs *During sham feeding experiments a subthreshold dose of histamine was* infused i v starting 1 hr before the sham feeding and continued for further 3 1/2 hrs The dose of histamine was choosen on the basis of the established dose response relationship

Insulin was administered i v in a dose of 0 2—0 4 I U per kg body weight and the acid response was recorded for 3 1/2 hrs



[illegible] mesenteri ve n to the inferior caval vein.

Results

Dogs that have been previously used for determining the effect of subthreshold amounts of gastrin on the sham feeding response (cf Olbe 1964 b have maintained their letter markin in the present study

Effect f exogenous histamine on sham feed ng response In 5 Pavlov pouch dogs (B E, M N O in which the acid sham feeding responses had been almost abolished by resection

TABLE II Dose response relationships in Pavlov pouch dogs for i.v. infusion of histamine dihydrochloride after resection of gastrin releasing regions (GRR) and after anastomosis of the superior mesenteric vein to the inferior caval vein (MCA). The response in expressed as the acid output during the 4th 15 min period of the infusion

Dog							
B	Dose of histamine						
	µg/kg/min	0	0.078	0.132	0.412	0.784	1.323
(1 exp.)	After GRR mEq	0	0	0.05	0.21	0.67	0.40
E	Dose of histamine						
	µg/kg/min	0	0.031	0.067	0.120	0.314	0.667
(4 expts.)	After GRR	0.02±	0.04±	0.04±	0.11±	0.45±	0.62±
	mEq±S.E.	0.01	0.02	0.02	0.06	0.18	0.20
(6 expts.)	After MCA	0.04±	0.05±	0.03±	0.06±	0.21±	0.48±
	mEq±S.E.	0.02	0.02	0.01	0.01	0.06	0.10
M	Dose of histamine						
	µg/kg/min	0	0.032	0.061	0.102	0.318	0.606
(4 expts.)	After GRR	0.01±	0.01±	0.01±	0.08±	0.74±	0.95±
	mEq±S.E.	0	0	0	0.03	0.13	0.14
N	Dose of histamine						
	µg/kg/min	0	0.016	0.048	0.092	0.155	0.483
(4 expts.)	After GRR	0.01±	0.01±	0.01±	0.10±	0.47±	0.62±
	mEq±S.E.	0.01	0.01	0.01	0.03	0.13	0.19
(5 expts.)	After MCA	0.09±	0.10±	0.12±	0.17±	0.42±	0.75±
	mEq+S.E.	0.02	0.03	0.04	0.03	0.06	0.08
O	Dose of histamine						
	µg/kg/min	0	0.296	0.630	1.130	2.963	6.296
(4 expts.)	After GRR	0.01±	0.01±	0.13±	0.32±	0.48±	0.49±
	mEq±S.E.	0	0.01	0.08	0.09	0.07	0.07
(3 expts.)	After MCA	0.04±	0.04±	0.15±	0.17 +	0.48	0.42±
	mEq±S.E.	0.03	0.01	0.05	0.02	0.08	0.04

of the gastrin releasing regions (Table I, Fig. 1 top curve, *cf.* Olbe 1964 b) an i.v. infusion of subthreshold amounts of histamine dihydrochloride markedly augmented — 5 to 43 times — the acid secretory responses to sham feeding (Table I, Fig. 1 3rd curve). The doses of histamine selected for the sham feeding experiments were just below those producing an acid response when infused alone (Table II). Accordingly the dogs never responded with acid secretion to the histamine infusion that preceeded the sham feeding (e.g. Fig. 1, 3rd curve).

Effect of anastomosis of the superior mesenteric vein with the inferior caval vein on the responses to sham feeding and to graded doses of histamine. Five Pavlov pouch dogs (B, E, F, N and O) in which resection of the gastrin releasing regions had almost eliminated the acid sham feeding responses (Table I, c.f. Olbe 1964 b) were subjected to an end-to-side mesenteric-caval anastomosis (MCA). All the vascular stomas were found patent on later

TABLE III Effect of portacaval transposition and subsequent resection of the antrum on the 3 5 hrs acid responses to insulin hypoglycemia in Pavlov pouch dogs (P) and Heidenhain pouch dogs (H)

		Mean acid responses to insulin hypoglycemia						
		Pre-portacaval transposition		Portacaval transposition			Antrum resection	
Dog	Dose of insulin IU/kg	mEq±S E	Number of expts	Fasting secretion mEq/hr	mEq±S E	Number of expts	mEq±S E	Number of expts
121 P	0 2	1 67±0 71	4	0 13	4 44±0 90	4	5 38±0 72	4
138 P	0 4	1 73 ±0 36	4					
		[1]1 01±0 32	4	0 79	5 55±0 83	4	3 92±0 41	4
160 P	0 2	0 39 ±0 08	4	0	1 28±0 08	4	1 25±0 12	4
120 H	0 4	0 06 ±0 05	2	0 03	0 09±0 05	3		
131 H	0 2	0 06	1	0 29	0 24±0 01	2		

[1] After additional duodenectomy

section The shunt markedly enhanced the acid sham feeding responses — 7 to 20 times (Table I, Fig 1 bottom curve) The augmentation produced by MCA was below that produced by subthreshold amounts of histamine before the vein anastomosis (Table I) except for dog E in which an unusually low dose of histamine was given pre-MCA (Table I and II)

Fasting acid secretion — ranging from 0 01 to 0 09 meq per 15 min — appeared after the vein anastomosis in most of the experiments on dogs B, N and O (e g Fig 1, bottom curve) and occasionally in dogs E and F

The dose-response relationship for histamine remained unchanged after MCA (Table II)

Effect of portacaval transposition on the acid responses to sham feeding and insulin hypoglycemia In 3 Pavlov pouch dogs (121, 138 and 160), one of which had been duodenectomized (138), portacaval transposition increased the acid responses to i v injected insulin (Table III) This increase was significant in 2 dogs ($p < 0\ 01$ in dogs 138 and 160 according to the Student *t* test) The post-transposition responses were not significantly altered by subsequent resection of the antrum (Table III)

Two Heidenhain pouch dogs (120 and 131) did not respond to injected insulin either before or after portacaval transposition (Table III) The post-transposition fasting secretion present in dog 131 vanished following the insulin injection

The sham feeding responses in 2 Pavlov pouch dogs L and 160) increased significantly (p 0 01) according to the Student *t* test) after portacaval tran

TABLE IV Effect of portacaval transposition and subsequent resection of the antrum on the 3 5 hrs acid responses to sham feeding in a Pavlov pouch dog (160) and a Heidenhain pouch dog (131)

	Mean acid responses to sham feeding						
	Pre portacaval transposition		Portacaval transposition			Antrum resection	
Dog	mEq±S E.	Number of expts	Fasting secretion mEq/hr	mEq±S E	Number of expts	mEq±S E	Number of expts
160	1 12±0 16	4	0 09	2 27±0 24	5	2 18±0 32	7
131	0 02±0 01	4	0 17	0 42±0 04	4		

and IV) In dog L the antrum had previously been resected and in dog 160 subsequent resection of the antrum did not change the sham feeding response (Table IV)

Sham feeding of a Heidenhain pouch dog (131) after portacaval transposition did not result in any increase of the acid secretion present under fasting conditions (Table IV)

Discussion

The direct vagal action on the gastric HCL secreting glands elicited by physiological means is an inefficient secretory stimulus since sham feeding during 10 min has been shown to produce an insignificant acid secretion in Pavlov pouch dogs after resection of the gastrin releasing regions (Olbe 1964 b) An acid sham feeding response appeared in these dogs on concomitant injection of subthreshold amounts of gastrin Thus the direct vagal action physiologically requires gastrin to result in a significant gastric acid secretion and this requirement can be supplied by the vagal release of gastrin (for ref see Olbe 1964 a) The cooperation between the direct vagal action on the gastric HCl secreting glands and gastrin constitutes a true potentiation according to the Gaddum criteria (Gillespie and Grossman 1964) on the reasonable assumption that sham feeding during 10 min represents maximum physiological activation of the vagi

The present study showed that the quality to potentiate the direct vagal action on the gastric HCl decreting glands is not characteristic of gastrin but a property shared with other humoral secretory stimuli (Fig 1) Injection of subthreshold amounts of histamine as well as mesenteric-caval anastomosis produced significant acid sham feeding responses in Pavlov pouch dogs devoid of their main sources of endogenous gastrin (Table I) The potentiation of the direct vagal action by histamine agrees with the facts that the secretory effect of histamine has been enhanced by a cholinergic disten

sion reflex (Grossman 1961) as well as potentiated by cholinergic drugs (Gillespie and Grossman 1964)

The cause of the augmented sham feeding response after mesenteric-caval anastomosis is open to question The present preparations and previously published observations eliminate gastrin from the discussion Thus in the present dogs with mesenteric caval anastomosis, the main sources of endogenous gastrin were resected and blood was shunted around the liver only from the intestines distal to the midportion of the duodenum Also the augmented acid responses to sham feeding and insulin hypoglycemia following portacaval transposition in Pavlov pouch dogs were not influenced by antrum resection Furthermore it has been previously shown that gastrin produces similar acid responses by portal and systemic injection (Olbe 1960, Gillespie and Grossman 1962)

Histamine liberated from the intestines has been suggested to cause the acid hypersecretion following portacaval transposition by escaping inactivation in the liver (Irvine *et al* 1959, Silen and Eiseman 1959 and 1961) The augmented sham feeding responses after the mesenteric caval anastomosis in the present study can be fully explained by subthreshold amounts of histamine being liberated into the systemic circulation from the non fed intestines (Table I Fig 1) However, such an interpretation it not easily reconsiled with the increased fasting secretion reported after portacaval transposition (Clarke *et al* 1958, Silen and Eiseman 1959) and found in the present study On the other hand a liberation of histamine in suprathreshold amounts from the intestines does not agree with the fact that the sham feeding responses potentiated by subthreshold amounts of histamine pre MCA exceeded the post MCA sham feeding responses in most dogs (Table I) despite that these dogs frequently had fasting secretion following the mesenteric-caval anastomosis As an alternative explanation the secretory agent from the unfed intestines may be a peptide of a constitution similar to gastrin or parts of gastrin (cf Tracy and Gregory 1964) No evidence has been obtained in the present study that vagal activation may release the intestinal secretory agent in an [illegible]

This investigation has been supported by grants from Karolinska Institutet Statens Medicinska Forskningsråd Y 190 Svenska Sällskapet for Medicinsk Forskning and Svenska Livförsakringsbolags Namnd for Medicinsk Forskning

References

CHILD C G D BARR G R [illegible] portacaval transposition in d [illegible]

CLARKE J S R S OZERAN J [illegible] portacaval shunt. *Ann Surg* [illegible]

CLARKE J S M HOFFS and S EL FARRA The effect of portacaval transposition on Heiden [illegible]

[illegible] 40—747

[illegible] *J appl* [illegible]

GILLESPIE I E and M I GROSSMAN Gastric secretion of acid in response to portal and system ic ven us injection of gastrin *Gastroenterology* 1962 *43* 189—19?

GILLESPIE I E and M I GROSSMAN Potentiation between urecholine and gastrin extract and between urecholine and histamine in the stimulations of Heidenhain pouches. *Gut* 1964 *5* 71—76

Grossman, M I, Stimulation of secretion of acid by distention of denervated fundic pouches in dogs *Gastroenterology* 1961 *41* 385—390

Irvine, W T, H L. Duthie, H D Ritchie and N G Waton, The liver's role in histamine absorption from the alimentary tract *Lancet* 1959 *I* 1064—1069

Olbe, L., Esophageal cannula dog a simple mode of preparation for sham feeding experiments *Gastroenterology* 1959 *37* 460—462

Olbe, L, Influence of portacaval transposition on gastric secretion in dogs *Acta physiol scand* 1960 Suppl. 175 110—111

[illegible]

Olbe, L, Effect of resection of gastrin releasing regions on acid response to sham feeding and insulin hypoglycemia in Pavlov pouch dogs *Acta physiol scand* 1964 b *62* 169—175

Silen, W and B Eiseman, The nature and cause of gastric hypersecretion following portacaval shunts *Surgery* 1959 *46* 38—47

Silen W and B Eiseman Evidence for histamine as the agent responsible for gastric hyper [illegible]

[illegible] structurally related to gastrin I *Nature* (Lond) 1964 *204* 935—938

Uvnäs B, The part played by the pyloric region in the cephalic phase of gastric secretion *Acta physiol scand* 1942 Suppl 13

Acta physiol. scand. 1966. 66. 481—483

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Histidine Induced Secretion in the Pyloric Ligated Rat Stomach

By

Toini Räsänen

Received 28 November 1965

Abstract

Rasanen T *Histidine induced secretion in the pyloric ligated rat stomach* Acta physiol scand 1966 66 481—483 — The amount and the pH of the gastric juice in the 1 hr pyloric ligated rat stomach were measured The amount and the hydrogen ion concentration of the gastric juice were greater when the rats were injected with histidine than with histamine The pretreatment with 5 × 1 mg of dexamethasone at 12 hr intervals degranulated the mast cells of the gastric mucosa and increased the pH in the gastric juice The difference of the secretory activity in histidine or histamine injected rats disappeared after the dexamethasone treatment The results suggest that the gastric parenchyma is stimulated immediately with histamine formed from histidine and stored in the gastric mucosal mast cell organ

After the exhaustion of the gastric mucosal mast cell organ with dexamethasone the acid secretion in the pyloric ligated rat stomach decreases The secretion can be restored however by giving a large amount of exogenous histamine (Rasanen and Haikonen 1965) It has been observed that mast cell granules are rich in histidine decarboxylase (Schayer 1965) The suspension of a mast cell tumour is capable of converting histidine to histamine (Lindell *et al* 1959) which also occurs in the tissue culture of mast cells Green and Day 1960

The activity of histidine decarboxylase increases in the different tissues during shock (Schayer 1960) It is possible that during pyloric ligation the formation of histamine in the gastric mucosa is activated and depends on the quantity of the mucosal mast cell granules In the following study the rats were injected either with histidine or with histamine the quantity and the acidity of the gastric juice of in the pyloric ligated rat stomach was then analyzed The effect of the degranulation of mucosal mats cells with dexamethasone on the secretory stimulation of histidine and histamine was also studied

TABLE I The amount and pH of gastric juice of the 1 hr pyloric ligated rat stomach in the dexamethasone treated and untreated animals after the injection of histamine or histidine

Treatment	No	Quantity	pH
Dexamethasone + histamine	10	5 10 ± 1 21 ml	3 91 ± 0 18
Dexamethasone + histidine	10	6 07 ± 0 58 ml	3 69 ± 0 60
Histamine	10	4 00 ± 0 53 ml	3 26 ± 0 26
Histidine	10	7 06 ± 0 71 ml	2 01 ± 0 09

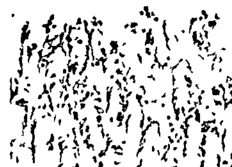


Fig 1

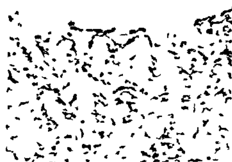


Fig 2

Fig 1 Gastric mucosal mast cells rich in metachromatic granules in the intact rat Magnification 500 ×

Fig 2 Nearly total disappearence of the metachromatic granules of the gastric mucosal mast cells after dexamethasone treatment Magnification 500 ×

Method

5 months old male rats weighing 215—400 g of the Dawley Spraguestrain were used in the study The rats adopted to the laboratory conditions in two weeks receiving mixed diet and

n ected ... e last ... after ... e or ... the

gastric mucosa

Results

The amount of the gastric juice in the pyloric ligated stomachs was greatest in the histidine injected rats (10 animals) differing clearly ($P < 0\ 01$) from that found in *histamine injected rats (10 animals)* (Table 1)

The concentration of the hydrogen ions was significantly higher in the former than the latter ($P < 0\ 001$) The pH in the gastric juice of dexamethasone pretreated rats

(10 animals in both groups) was higher than in those receiving histamine ($P < 0.01$) or histidine only ($P < 0.001$). After the dexamethasone treatment, the difference in the concentration of the hydrogen ion in the gastric juice of rats injected with histamine or with histidine, disappeared.

The microscopical examination revealed nearly total loss of granulation in the mucosal mast cells after dexamethasone treatment (Fig 1—2).

Discussion

According to previous studies, the superficial part of the rat stomach mucosa contains on an average 50,000—80,000 mast cells per cubic mm of tissue (Rasanen 1964). Glucocorticoid treatment decreases the histamine content in the rat gastric mucosa, by decreasing the number of granulated mast cells (Foley and Glick 1962). Dexamethasone very effectively degranulates gastric mucosal mast cells (Rasanen 1962).

After the dexamethasone treatment in the pyloric ligated rat stomach the acid secretion decreases but recovers after administration of lage amount of exogenous histamine (Rasanen and Haikonen 1965).

The mast cell granules contain an enzyme which probably converts the histidine to histamine. It is activated in shock of different kinds (Schayer 1960). Possibly histidine decarboxylase becomes activated *in the gastric mucosal mucosal mast cells during the* pyloric ligation. The formation of new histamine in the gastric mucosa of histidine injected rats possibly leads to greater local stimulation of the parenchymal cells than an injection of the same amount of exogenous histamine.

The degranulation of the mucosal mast cells probably causes the inactivation of the histidine decarboxylase and the inhibition of secretion in the pyloric ligated stomach. The loss of the histamine stores in the gastric mucosa after the dexamethasone treatment also inhibits the stimulating effect of the pyloric ligation as well as of small quantities of exogenous histamine. The glycogenic effect of dexamethasone does not inhibit the acid secretion because large amounts *of histamine restore secretion, and after a single* injection of dexamethasone the secretory capacity of the gastric parenchyma increases — in the phase of the mast cell degranulation (Rasanen and Haikonen 1965) and possibly of the local histamine liberation in the gastric mucosa.

This study is sponsored by the Sigrid Jusélius Foundation

References

Fo [illegible]

Gr [illegible]

HAIKONEN H and RASANEN T. Hydrogen ion concentration in the gastric juice after pyloric ligation in dexamethasone-treated rat. *Acta physiol scand* 1965 *65* 378—382

LINDELL S E, H RORSMAN and H WESTLING. Formation of histamine in a canine mastocytoma. *Experientia* (Basel) 1959 *15* 31—37

RÄSANEN T. Effects of dexamethsone, prednisolone and cortisol on the mast cells and tissue [illegible] 1962 *41* 432—436

[illegible]

SCHAYER R W. Relationship of induced histidine decarboxylase activity and histamine synthesis to shock from stress and from endotoxin. *Amer J Physiol* 1960 *198* 1187—1192

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Firing with Multiple-Spike Discharges in the Slowly Adapting Stretch Receptor Neuron of the Lobster

By

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Received 7 December 1965

Abstract

GRAMPP W *Firing with multiple-spike discharges in the slowly adapting stretch receptor neuron of the lobster* Acta physiol scand 1966 66 484—494 — The phenomenon of firing with multiple- [illegible]

took place during the course of three different types of after-depolarizations which succeeded the first and, sometimes a number of the subsequent impulses of the multiple-spike discharges From its reproducibility and reversibility it is concluded that the phenomenon of firing with multiple-spike discharges reflects a (reversible) modification of the cell's excitatory state due to changes of the conditions of the external milieu

Under conditions which simulate those of their normal environment most cells out of a population of slowly adapting stretch receptor neurons of crayfish or lobster fire with single action potentials (Wiersma *et al* 1953 Eyzaguirre and Kuffler 1955a Only a few cells fire with discharges consisting of several spikes following each other in a rapid succession (grouped discharges double discharges, repetitive discharges rhythmic-periodical discharges) (Eyzaguirre and Kuffler 1955b, Edwards and Ottoson 1958) However it has been observed by Florey (1956) that it is possible to make the slowly adapting stretch receptor neuron of crayfish shift from firing with single spikes to firing with grouped discharges by raising the temperature above 30° C This observation has been confirmed by Burkhardt 1959) who also noticed that the spike number of the grouped discharges increases and decreases in a rather regular way with rising and falling temperature Under constant environmental conditions a change in the number

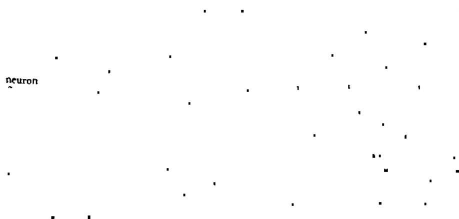
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of spikes per discharge can be provoked also by altering the stimulation intensity (Eyzaguirre and Kuffler 1955b)

Similar effects of the temperature and of the strength of stimulation on the spike number of the discharges have been observed also in the slowly adapting stretch receptor neuron of lobster (Grampp 1963) Since it was found in preliminary experiments that these effects could be reproduced in all cells studied they were made the subject of a systematic investigation

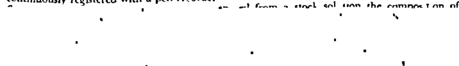
Material and methods

Preparation Excised abdominal stretch receptor organs of the lobster (*Homarus gammarus*) (Alexandrowicz 1951) were used The organs were attached to the forceps of a preparation hold-



neuron

continuously registered with a pen recorder



intracellularly which gives rise to an outward transmembrane current flow Current applied to a cell which produces an inward transmembrane current flow is called anodal current

The term multiple-spike *discharge* or *specified*, two-spike discharge three-spike discharge etc) will be used to denote a discharge which independently of the kind intensity and duration of the eliciting stimulus consists of several or a specified number of spikes in a rapid succession

Results

Firing with multiple-spike discharges All the slowly adapting stretch receptor neurons studied were firing no matter how they were stimulated stretch of the receptor muscle, injection of cathodal current antidromic stimulation with single action potentials at

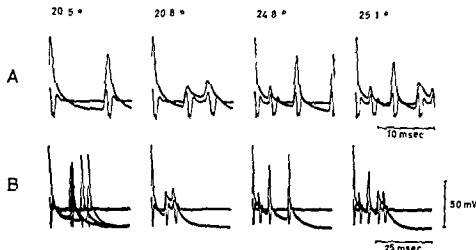


Fig 1 Stretch evoked discharges of a cell recorded simultaneously intrasomally (upper traces) and extraaxonally (lower traces) at a fast sweep A and a slow sweep B at temperatures critical for ch
record at 20 °
The temperat
deflections of
tential change
by the first action potential of each discharge

temperatures below and with multiple spike discharges at temperatures above a critical value This value was different for different cells, it happened that it occurred at about 15° C (Fig 4) or at about 30 C (Fig 5), but usually it occurred between 20 and 25 C (Fig 1 and 2)

In response to a steady depolarization due to stretch of the receptor muscle or intracellularly injected cathodal current all cells were able to fire repetitively with multiple spike discharges just as with single spike discharges at lower temperatures for indefinitely long periods of time Usually if the spike number was small (< 4—8) it was the same in successive discharges of a repetitive activity, provided the experimental conditions were constant However if the spike number was larger it varied considerably from discharge to discharge even when there were no changes whatever of the experimental conditions

The effect of the temperature on the spike number of the discharges at constant stimulation intensity In each cell a gradual increase of the number of impulses making up the multiple spike discharges could be provoked by raising the temperature above the value critical for shift from firing with single action potentials to firing with multiple-spike discharges This increase took place at certain critical increasingly higher temperatures by instantaneous addition of a number of impulses to the already existing spikes of the discharges In many cases this increase was regular and predictable as long as the number of impulses of the discharges was small Thus in one group of the cells the number of impulses increased by one at a time until the discharges consisted of about 5 impulses (Fig 1) In another group of cells the number of impulses increased from one to two and then by two at a time until the discharges consisted of 6—10

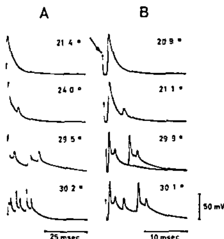


Fig 2

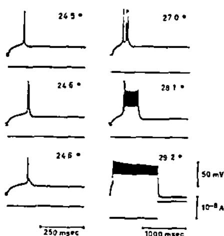


Fig 3

(A) and 10 msec for all other records

Fig 3 Discharges of a cell (upper traces) evoked by intrasomally injected cathodal square current pulses (lower traces) recorded intrasomally at different temperatures Time calibration 1 000 msec for record at 29 2° C and 250 msec for all other records

impulses (Fig 2 A) Each of these changes in spike number occurred at a fixed temperature (see below) The further increase with rising temperature of the number of impulses per discharge was irregular in both groups of cells, i e the changes in spike number did not occur at fixed temperatures and the number of impulses added each time was unpredictable and often large In this way the spike number of the discharges often grew to several hundred as the temperature was raised through a relatively narrow interval which in most cases occurred around and above 30 C (Fig 3, *27 0—29 2° C*) After this there was no further increase but sometimes there was even a slight decrease of the spike number of the discharges as the temperature rose further It never happened though even at the highest temperatures tried (37—38° C) that the discharge activity stopped altogether as may be the case in the slowly adapting stretch receptor neuron of crayfish (Burkhardt 1959) — In some cases the increase in spike number of the discharges was irregular from the very beginning However as was true for the behaviour of the types of cells described above the number of impulses added each time usually was small at lower but became large at higher temperatures

A decrease in the spike number of the discharges could be provoked by lowering the temperature In the great majority of cells the course of events taking place during this decrease was that of the increase in exactly reverse order Thus in all cells investigated the number of impulses making up the discharges could be increased and decreased again any number of times just by repeatedly raising and lowering the temperature

As long as the increase or decrease of the number of impulses per discharge was regular it was possible to provoke a uniform change in spike number in all the dischar-

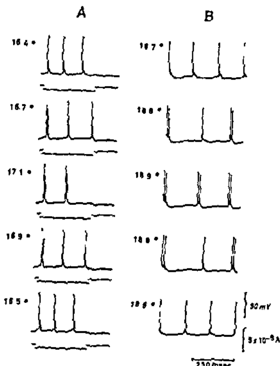


Fig. 4. Intrasomal recording of repetitive activities of a cell evoked by intrasomal injection of cathodal square current pulses of constant amplitudes (lower traces in A) after relaxation of the receptor muscle A and by constant stretch of the muscle, B. The activities were registered as the temperature was raised and lowered through intervals critical for shifts from firing with single-spike to firing with two spike discharges and back to firing with single-spike discharges

ges of a repetitive activity. Each change from firing with *n*-spike discharges to firing with $(n + 1(2))$-spike, or $(n - 1(2))$-spike discharges took place as the temperature rose, or fell, respectively through a certain critical interval. In different cells these intervals occurred at quite different temperatures, just as was found to be the case with the interval critical for change from firing with single action potentials to firing with multiple-spike discharges. However, in the individual case the position of each interval on the temperature scale was remarkably constant during long periods of time. Usually an interval did not move more than a few tenths of a degree per hour (*cf.* Fig. 5). The average width of the interval was 0.2° C (S.D. = ± 0.1) (Fig. 4 B). The liminal values of any interval were given by those temperatures at which the number of impulses was altered in only a few, and in all discharges of a repetitive activity, respectively. Within each interval the spike number usually varied between those of the discharges fired at temperatures below, and above the interval, respectively (Fig. 4). The proportion of the discharges having the greater number of impulses increased gradually as the temperature was approaching the higher liminal value of the interval, a corresponding increase of the proportion of discharges with the lower spike number occurred as temperature was falling towards the lower liminal value. Some cells in one or more of the critical intervals fired discharges the spike number of which was not related to those of the discharges which occurred at temperatures below, or above the interval(s) respectively.

A rapid and small variation of the temperature within, or near a critical interval was seen to cause a temporary change in the spike number of the discharges (Fig. 6,

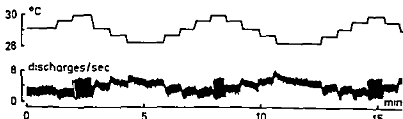


Fig 5 The effect of temperature steps (upper trace) on the discharge frequency and the number of spikes per discharge (lower trace) The discharge activity was evoked by constant stretch of the receptor muscle and recorded extraaxonally Pen deflections of double length indicate two-spike discharges

indicated by arrows) This reaction occurred initially during the phase of adaptation following the abrupt change of the discharge frequency due to the temperature step (*cf* Burkhardt 1959)

observed which bore a close resemblance to what previously has been observed in the slowly adapting stretch receptor neuron of crayfish (Burkhardt 1959) i e as the temperature rose through a certain interval every second impulse in a train of originally regularly spaced single-spike discharges moved increasingly closer to its forerunner until finally the cell was firing with regularly spaced two-spike discharges At higher temperatures successive two-spike discharges moved together two and two to make regularly spaced four spike discharges At still

The effect of the stretch stimulation intensity on the spike number of the discharges In the great majority of cases a change from firing with *n* spike to firing with $(n + (-)\, a)$ spike discharges took place at higher temperatures when the stretch stimulation intensity was high rather than low or negligible as in relaxed electrically stimulated cells (Fig 6) In just a few cells and only as long as the spike number of their discharges was small ($<$ 3—5) firing with discharges having a certain number of impulses occurred at lower temperatures because the stretch of the receptor muscle was strong rather than weak

From this follows that at any constant temperature preferably when the latter was kept near a value critical for a change of the spike number of the discharges it was possible to alter not only the discharge frequency but also the number of impulses of the discharges by varying the stretch stimulation intensity Thus in most cases and always when the spike number of the discharges was large ($>$ 8—10) a number of the impulses making up the multiple spike discharges could be abolished by stretching and added by relaxing the receptor muscle The opposite effect of the stimulation intensity on the spike number was seen only in a few cells when firing with discharges consisting of a small number of impulses The number of spikes abolished or added in this way was small (1—4) when the temperature was such that a variation of it also was causing small (and regular) changes in the spike number of the discharges, at

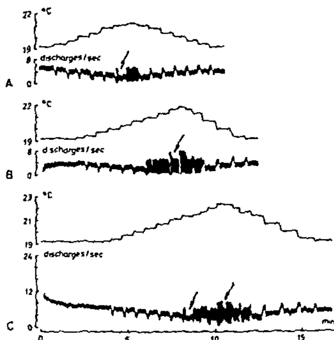


Fig. 6. The effect of temperature steps (upper traces) on the discharge frequency and the number of spikes per discharge (lower traces) at different stimulation intensities. The stretch of the receptor muscle was decreased from A to B and increased from B to C. The discharge activity

after an abrupt variation of the temperature near a temperature interval critical for change [illegible] the spike number of the discharges

higher temperatures a corresponding variation in muscle length usually resulted in the abolition or addition of a large number of impulses per discharge.

In the individual discharges each stretch-induced change of the spike number took place in all-or-nothing fashion. In order to alter the spike number in all discharges making up a repetitive activity the stretch stimulation intensity had to be changed by a certain minimal amount. A temporary change in the spike number of the discharges was seen initially during the adaptation of the discharge frequency occurring after small and abrupt changes of the muscle length (cf. Florey 1956, Burkhardt 1959).

The effect of the temperature and stretch stimulation intensity on the impulse frequency within multiple-spike discharges. In some cells at temperatures just above the critical value for change from firing with single-spike to firing with multiple-spike discharges the average impulse frequency within the latter could be as low as 50—70/sec. However, in most cases under corresponding conditions the firing rate within the discharges never went below 100/sec. Irrespective of its lowest value the average impulse frequency within the multiple spike discharges fired by any particular cell always increased with rising temperature. It finally assumed values of about 500/sec in discharges consisting of many (> 10—20) impulses. However, for temperatures at

and above 30° C in the multiple-spike discharges of many cells time intervals were seen between successive impulses which corresponded to firing frequencies of about 800 sec (Fig 2 B, 30 1° C 'interval bet [illegible]

rat [illegible]

ature by increasing the stretch stimulation intensity In those few cells in which an intensification of the stretch of the receptor muscle resulted in the addition of extra spikes to the discharges it also gave rise to an increase of the impulse frequency within the latter

The multiple spike discharge When recorded with a wire electrode placed against the axon in liquid paraffin multiple spike discharges appeared as trains of diphasic, or triphasic, impulses in all cells investigated In each individual case these impulses did not differ in time course from each other, or from single impulses, provided the firing frequency within the multiple spike discharges was not too high (Fig 1, *20 5° C*) However, a decrease of the amplitude of the negative phase of a number of extra spikes in each discharge took place, when the firing rate exceeded 200—250/sec (Fig 1, *20 8—25 1° C*)

A greater variability in time course was seen in intrasomally recorded multiple-spike discharges This was because of the following facts

(1) In the soma successive spikes were able to reach full height only when the time intervals between them did not grow shorter than about 10 msec If they did so, the spike amplitudes became reduced The degree of reduction increased with the degree of shortening of the duration of the intervals (Fig 1 B, *20 5° C*) A decrease of the rates of rise and fall was seen to take place in those impulses the amplitudes of which had been reduced below a certain value (Fig 1 and 2) Since in most burst discharges a number of spike intervals were shorter than the above stated liminal value, it follows that in intrasomal recordings of these discharges a corresponding number of the extra spikes had a smaller amplitude and lower rates of rise and fall than the first full-sized action potential. In most multiple-spike discharges the length of successive impulse intervals varied considerably This explains the differences in time course between the extra impulses of intrasomally recorded discharges In discharges registered with a wire electrode placed against the axon in paraffin such differences were less obvious (Fig 1) Partly this was because of the mode of recording but mainly because the impulse frequency usually was too low for an appreciable reduction of the heigth of axonal impulses (*cf* Grampp 1966a)

(2) The initiation of the extra impulse(s) of the multiple-spike discharges took place only during the course of different types of prolonged depolarizations or slow repolarizations which succeeded the first and sometimes a number of the subsequent impulses of the discharges These particular depolarizations have been called after-depolarizations because none of them was seen to occur unless after a preceding action potential no matter how the latter was elicited

Three different types of these after polarizations could be identified (a *The slow after-depolarization* This kind of after-depolarization appeared as a slow wave of depolarization which followed the first full-sized action potential of the discharge prominent in Fig 2 A *30 2 C* and Fig 3 *28 1—29 2° C* The duration of the slow after-depolarization increased with rising temperature it could become as long as several hundred milliseconds or even several seconds. (b) *The fast after-depolarization* This after-depolarization immediately succeeded the initial and replaced the later part of the repolarizing

phase of the first full-sized, and of a number of the subsequent moderately reduced action potentials of the discharge (e g Fig 2 A, *24 0—30 2° C*, after the large spikes) (c) *The labile after-depolarization* This after-depolarization occurred after small spikes of a type indicative of the blockage of the conduction of the active process. Multiple-spike discharges in which the first impulses was a small spike could be elicited by stimulating a relaxed cell antidromically at temperatures above a critical value (*cf* Grampp 1966a) — The described after-depolarizations developed at temperatures, and at rates which were different for different cells. This was seen to result in dissimilarities in time course between the intrasomally recorded multiple-spike discharges of different cells, even when they consisted of the same number of impulses, and were registered under identical experimental conditions (A more detailed description of the different types of after-depolarizations is given elsewhere, Grampp 1966b, c.)

Multiple-spike discharges which were evoked by stretch of the receptor muscle, or by intracellularly injected cathodal current were succeeded, like single action potentials by an after-hyperpolarization (Fig 3, *24 5—28 1° C*, and Fig 4) During the course of the latter the membrane was hyperpolarized not in relation to the resting level, but in relation to the potential level of the steadily depolarized cell (Eyzaguirre and Kuffler 1955b)

Discussion

Even though most of the slowly adapting stretch receptor neurons of the lobster seem to fire with single action potentials under normal environmental conditions, firing with multiple-spike discharges is not regarded as an unphysiological phenomenon indicating cellular injury due to harmful experimental procedures This view is based on the following facts and considerations (1) Firing with multiple-spike discharges was seen to occur independently of the mode of stimulation It could be recorded with intra- as well as with extracellular techniques Multiple-spike discharges are known to be fired by the slowly adapting stretch receptor neuron of crayfish also when the former is kept in the blood of the animals (Burkhardt 1959) These findings indicate that neither the stimulation and recording techniques nor the composition of the artificial solution are the essential causes of the generation of multiple-spike discharges in the present material 2 It has been impossible to produce irreversible changes in the physiological behaviour of the cell by raising the temperature up to 37—38° C, the highest values tested see Grampp 1966a, Fig 2 Thus the slowly adapting stretch receptor neuron of the lobster is able to tolerate temperatures several degrees higher than those at which firing with single action potentials is replaced by firing with multiple-spike discharges. This tolerance of high temperatures in not quite unexpected in view of the fact that at least some of the aquatic decapods do not react until the temperature is raised up to about 40 C (Barber 1960 3 The constancy of the time course of successive multiple spike discharges in a repetitive activity and the fixedness on the temperature scale of the intervals critical for change of the spike number of the discharges indicate that neither firing with multiple-spike discharges as such nor the temperature at which it takes place produces progressive changes in the physiological status of the cell From this is evident that the stretch receptor neuron can adapt itself to conditions which differ from those of its normal environment 4 In some cells firing with multiple-spike discharges was seen to occur at temperatures normal for the environment of the living animal (*cf* Eyzaguirre and Kuffler 1955b Edwards and Ottoson 1958) In these cells

like in other ones which fired with multiple-spike discharges at higher temperatures, a shift to firing with single action potentials could be provoked by lowering the temperature below a certain value From this follows that the generation of multiple spike discharges is not absolutely dependent on 'unphysiologically' high values of the temperature

It is concluded therefore, that the temperature-dependent shifts from firing with single action potentials to firing with multiple spike discharges and *vice versa* are signs of reversible modifications of cellular properties which determine the time course of the excitability and polarization changes that take place subsequent to an active depolarization of (parts of) the neuronal membrane The effects of the temperature on the duration of the refractory period as well as on the rates of the preceding ionic permeability changes that give rise to the spike potential are supposed to be essentially the same in the stretch receptor neuron as in other materials investigated (for references see Tasaki and Spyropoulos 1957) The dependence on the temperature of the potential changes that follow a regenerative activity is evident from the fact that with rising temperature the final phase of repolarization in each discharge became delayed more and more as a result of the development of one of, or one of several possible combinations of three different types of after-depolarizations

These after-depolarizations were absent or small in single-spike discharges but they were always present in multiple spike discharges It is likely, therefore, that they are the generators of the extra spikes of the latter type of discharge It is possible then that the changes with varying temperature of the spike number of the discharges and of the impulse frequency within the latter are due to temperature induced modifications both of the duration and amplitude of the prolonged depolarizations and of the lengths of the refractory periods The dependence on the (stretch) stimulation intensity of the spike number and the rate of firing within the discharges indicates that the time courses of the after-depolarizations are determined (1) by the degree of steady depolarization and (2) by membrane characteristics that evidently are functions of the polarization (delayed rectification *cf* Edwards *et al* 1963) and/or of the mechanical deformation of the membrane (*cf* Terzuolo and Washizu 1962) The background depolarization may also influence the rate of recovery after a preceding activity (*cf* Chapman 1964)

From the fact that in different cells the spike number of the discharges changed at different temperatures and at different rates it has to be concluded that there is a biological variation among stretch receptor neurons with respect to those properties that determine the time course of the excitability and polarization changes after an active depolarization In a few cells these properties and their constellation the configuration as it was expressed by Eyzaguirre and Kuffler (1955b are such as to predispose to a rapid recovery cycle and to the generation of prolonged depolarizations already under normal environmental conditions However in most cells this particular excitatory state does not seem to arise until the configuration has been altered a certain minimal amount by raising the temperature

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References

ALEXANDROWICZ, J S, Muscle receptor organs in the abdomen of *Homarus vulgaris* and *Palinurus vulgaris* *Quart J micr Sci* 1951 *92* 163—199

BARBER, S B, Chemoreception and thermoreception In *The Physiology of Crustacea*, T H Waterman, Editor, Vol II, pp 109—131, Academic Press, New York and London, 1960

BURKHARDT, D, Die Erregungsvorgänge sensibler Ganglienzellen in Abhängigkeit von der Temperatur *Biol Zbl* 1959 *78* 22—61

CHAPMAN, R A, Lability of the action potential and the recovery cycle from isolated motor axons of the crab, *Carcinus* *Nature* (Lond) 1964 *203* 872—873

EDWARDS, C and D OTTOSON, The site of impulse initiation in a nerve cell of a crustacean stretch receptor *J Physiol* (Lond) 1958 *143* 138—148

[illegible] 119

EYZAGUIRRE, C and S W KUFFLER, Further study of soma dendrite and axon excitation in single neurons *J gen Physiol* 1955b *39* 121—153

[illegible] neuron of the lobster *Acta physiol scand* 1966a *66* Suppl 262

GRAMPP, W, Multiple-spike discharge evoking after-depolarizations in the slowly adapting stretch receptor neuron of the lobster I The labile and the fast after depolarization *Acta physiol scand* 1966b *67* In press

GRAMPP W Multiple spike discharge evoking after-depolarizations in the slowly adapting stretch receptor neuron of the lobster II The slow after-depolarization *Acta physiol scand* 1966c *67* In press

HAAPANEN L and D OTTOSON A frequency compensated input unit for recording with microelectrodes *Acta physiol scand* 1954 *32* 271—280

KANDEL E R W A SPENCER and F J BRINLEY JR Electrophysiology of hippocampal neurons I Sequential invasion and synaptic organization *J Neurophysiol* 1961 *24* 225—242

T[illegible] on the [illegible] 01—

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WIERSMA C A G, E FURSHPAN and E FLOREY Physiological and pharmacological observations on muscle receptor organs of the crayfish *Cambarus clarkii* Girard *J exp Biol* 1953 *30* 136—150

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The Effect of Vagal Stimulation on the Bronchial Tree in the Cat

By

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Abstract

SCHWIELER, G H *The effect of vagal stimulation on the bronchial tree in the cat* . physiol scand 1966 *66* 495—500

A method is described whereby bronchial diameter changes can be recorded in artificially vent lungs It is shown that the bronchi dilate during positive inflation When the peripheral ends cut vagal nerves are stimulated in the cervical region, this dilatation decreases and the bronchi be stiff It is concluded that this is an effect of bronchial smooth muscle contraction
The results are confirmed in spontaneously ventilating animals by bronchography and measurer of dynamic compliance

Opinions differ with regard to the efferent sympathetic and parasympathetic influ on bronchial smooth musculature Most authors have found indirect evidenc contraction of these muscles on stimulation of the vagi, but others report relaxa Similar contradictory results have been obtained on stimulation of the sympatl nerves (Macklin 1929, Wyss 1952 and Widdicombe 1963) Moreover, the metl mainly used for measuring changes in bronchial smooth muscle tone have l criticized by some authors It is generally assumed that contraction of the bronc muscles causes a decrease in the bronchial diameters with an increase in resist to air flow and that this can be measured as a change in the pressure-volume r tionship of an artificially ventilated lung (see e g Dixon and Brodie 1903, Kon and Rossler 1940, Daly and Mount 1951 and Kunzler 1962) However, Cro and Widdicombe (1961) maintain that the pressure-volume relationship is n sensitive to changes in stiffness of the total lung than to changes in air flow resista Whether an increase in lung stiffness is caused by vascular congestion, edema, alte air flow resistance or an increase in bronchial smooth muscle tone without a decre in the airway diameters, is not possible to determine with the usual methods (Wic combe 1963) Thus, a method by which the bronchial diameter may be measu from moment to moment throughout the respiratory cycle is required for inve gating possible effects on

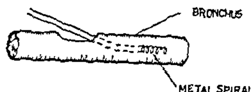


Fig 1 Graph showing how the metal spiral placed in a bronchus



Fig 2 Radiograph of the right inferior lobe to show where measurements of the bronchial diameters were made (arrows)

In the present paper such a method will be described By means of a capacitive transducer, the diametric changes in a bronchus which had been freed from its surrounding vessels and lung parenchyma were recorded The aim was to reinvestigate the effect of vagal stimulation on the bronchi

Methods

Adult cats were used They were anesthetized with pentobarbital (Nembutal Abbot) given intraperitoneally in a dose of 40 mg kg b w A cannula was inserted into the trachea as routine Both vagi were dissected free in the cervical region and cut just below the nodose ganglia The sympathetic trunks were separated from them and cut In the animals which were thoracotomized (see below) the upper 5 thoracic sympathetic ganglia were also removed bilaterally

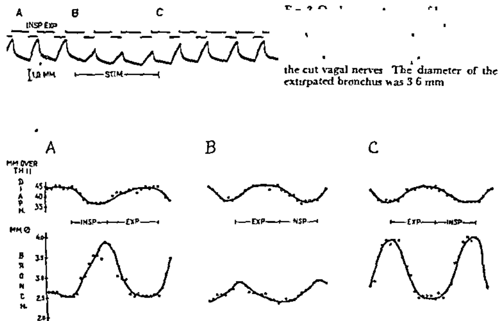
Stimulation was performed by s ramax mal shocks at frequencies of between 10 and 20 sec applied as far rostrally as possible on the peripheral ends of the cut vagi

In the first series of experiments performed on some 20 animals a capacitive transducer was used

etween a metal spiral s mated

in bronchial diameter These

Thoracotomy was performed on the right side The tracheal cannula was connected to a respirator (Model RU — 4M ENSCO) The inferior right lobe was freed fr m the pleural sheaths connecting it with the diaphragm and the



the cut vagal nerves The diameter of the extirpated bronchus was 3 6 mm

Fig 4 Graphs showing changes in bronchial diameter with changes in level of diaphragm as measured on radiographs taken with a frequency of 3 sec during a respiratory cycle a) before b) during and c) after stimulation of the peripheral ends of the cut vagal nerves

mediastinum It was then fixed with threads to prevent it from gross movements caused by respiration The pulmonary arteries and veins which supplied the lobe being investigated were tied at the lung hilus and dissected free about 2 cm distally This was done in order to prevent any possible influence from the pulmonary vessels on the bronchial diameters The lung parenchyma around the bronchus

(e g through mucus secretion or displacement of the transducer) was easily recognized on the oscilloscope screen The transducer was calibrated and exact values for the diametric changes could thus be obtained

In a second series of experiments bronchographic studies were performed on 6 spontaneously ventilating animals A catheter was introduced through the tracheal cannula and with the aid of an image amplifier its tip was placed at the origin of a lobar bronchus. 1 4—1 2 ml of Dionosil (GLAXO) was then injected into the lobe through the catheter In this way the bronchial tree of only one lobe was outlined with the contrast medium which simplified the measurements see below Exposures were taken with a frequency of 3 sec before during and after stimulation of the vagi The diameter of different bronchi was measured on each radiograph Since the bronchi may elongate during inspiration each measurement was made halfway between two ramifications and thus at identical levels on the bronchi under investigation Fig 2 Moreover the level of the diaphragm above a certain vertebra and thus the moment in the respiratory cycle was determined on each radiograph

Finally in a third series of experiments the dynamic compliance of the lungs was measured in 1 cats before during and after stimulation of the vagi Dynamic compliance is defined as "the ra the tidal volume to the change in pressure between the points of zero flow at the extremes of volume expressed in ml cm H_2O (Mead 1961 In order to measure the intrapleural pressu a cannula was inserted through

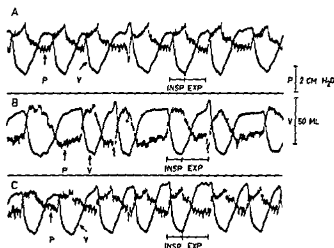


Fig 5 Oscilloscope tracing showing the variations in intrapleural pressure (P) and tidal air (V) during respiration a) before b) during and c) after stimulation of the peripheral ends of the cut vagal nerves The values for compliances were a) 22 0 b) 14 5 and c) 22 3 ml/cm H_2O

this was assumed to correspond with the turning points on the volume curves

Results

Capacitance measurements From the results obtained with the capacitive transducer, it is seen (Fig 3A) that the bronchus dilates by about half its diameter on positive inflation During stimulation of the vagi this dilatation decreases (Fig 3B) by between 50 % and 80 %, this varying in different experiments There is also a slight decrease in bronchial diameter at the end of passive deflation When stimulation of the nerves is interrupted, the bronchus dilates almost instantaneously on inflation as before (Fig 3C)

Bronchography The bronchographic investigations also show that, during normal breathing, the bronchi dilate by about half their diameters on inspiration (Fig 4A) During vagal stimulation this inspiratory dilatation diminishes (Fig 4B) by between 60 % and 100 %, this varying in different experiments A slight decrease in the bronchial diameters during the expiratory pause is also seen As soon as the stimulation is interrupted, the bronchi dilate in their normal fashion again (Fig 4C)

Dynamic compliance measurements During spontaneous breathing, the dynamic lung compliance is about 25 ml cm H_2O (22 0—31 5 ml/cm H_2O) When the vagal nerves are stimulated it decreases by about 30 % (16 %—39 %) On interruption of this stimulation the dynamic compliance increases to the same values as before (Fig 5A, B and C)

Discussion

The results of the present investigation show that, in adult cats, stimulation of the peripheral ends of the cut vagi in the cervical region causes a decrease in bronchial inspiratory dilatation Since the vessels and lung parenchyma around the investi-

gated brochus had been removed, and because of the rapidity of the response and its reversibility, it is concluded that this bronchial "stiffness" is caused by contraction of its smooth muscles

It may at first seem difficult to understand why earlier authors have obtained conflicting results in spite of the fact that they have used the same experimental methods (see discussions e g by Macklin 1929, Wyss 1952, and Widdicombe 1963) Since, however, these investigations were made on whole lungs, the following factors must be taken into consideration Firstly, the pulmonary vascular bed may have influenced the results, since the methods mainly used for estimating alterations in smooth muscle tone in the respiratory tract are based on measurements of the dynamic pressure volume relationship in artificially ventilated lungs As mentioned in the introduction, these methods seem to be more sensitive to changes in lung stiffness than to changes in airway calibre It is well known that both dilation of the lung vessels and increase in the pulmonary capillary pressure cause a decrease in compliance (Mead 1961) According to Daly (1961) the cervical vagosympathetic trunks send both vasoconstrictor and vasodilator fibres to the lungs It thus follows that the vascular bed might have affected the results of these investigations For the same reason a vascular factor must also be expected to have influenced the measurements of total lung resistance (Mead and Whittenberger 1953), used by Nadal and Widdicombe (1962) as an index for airway diameter Moreover, it is possible that the pulmonary vessels affect the bronchial diameters directly Thus, the results of measurements of fluid flow through the tracheo bronchial tree (Thornton 1932), with endobronchial balloons (Ellis and Livingstone 1935, Colgan 1964) and radiographically (Francis 1929, Sato 1936) are similarly open to criticism

Secondly the pulmonary sympathetic nerve supply might have influenced the results because of the varying routes of these fibres Very few authors have mentioned the level at which the vagal nerves were stimulated or whether or not they separated the sympathetic from the vagal nerves Daly and Mount (1951) have shown that sympathetic fibres may loop up the cervical vago sympathetic trunk to different levels before descending into the thorax Moreover, Sternschein (1922) and Kiss (1932) have demonstrated connections between the vagal and sympathetic nerves There is thus some reason to believe that different numbers of sympathetic fibres could have been stimulated simultaneously with the vagal fibres in some experiments This may be of importance since the vagal and sympathetic influence on the bronchi are considered to be antagonistic

Finally it is evident from the above that it has not been possible with any of the earlier methods to elucidate whether an increase in bronchial smooth muscle tone causes a decrease in diameter of the bronchi and increase in their elasticity or both

In the present work the vessels and lung parenchyma around the bronchus under investigation were removed Thoracotomy and artificial ventilation w[illegible] therefore necessary It was suspected that the pressure relationships u[illegible] circum tances might [illegible] chaviour of the bronchial tree Ho[illegible]

respect to the inspiratory dilatation, this did not occur as seen from the bronchographic investigations, during which the animals were breathing spontaneously

It was considered possible that the decrease in inspiratory dilatation during vagal stimulation, observed with these methods might alter the dynamic lung compliance This was tested, and it was shown that the compliance decreased by about 30 %

In the bronchographic investigations and compliance measurements a vascular influence on the bronchial tree cannot be excluded Whether there were any significant quantitative differences caused by such influence, was not investigated here It is only claimed that the principal results are the same when the thorax the pulmonary vessels and the thoracic sympathetic nerve supply are intact

The costs for this investigation have been partly defrayed by 'Svenska Sällskapet for Medicinsk Forskning

References

COLGAN, F J Relationship of changing bronchial calibre to respiration in the dog *J appl Physiol* 1964 *19* 803—811

CROSFILL, M L and J G WIDDICOMBE Phys cal characteristics of the chest and lungs and the work of breathing in different mammalian species *J Physiol* (Lond) 1961 *158* 1—14

DALY I DE B The nervous control of the pulmonary circulation *Naunyn-Schmiedeberg s Arch exp ath Pharmak* 1961 *240* 431—445

M DE B and L E MOUNT The origin course and nature of bronchomotor fibres in the cal sympathetic nerve of the cat *J Physiol* (Lond) 1951 113 43—62

ON W E and T G BRODIE Contributions to the physiology of the lungs Part 1 The bronchial muscles their innervation and the action of drugs upon them *J Physiol* (Lond) 1903 *29 97—173*

ELLIS M P and A E LIVINGSTONE A method of directly recording changes in the calibre of the bronchi *J Physiol* (Lond) 1935 *84* 223—231

EULER, C V and H W FRITTS Quantitative aspects of respiratory reflexes from the lungs and ches walls of cats *Acta physiol scand* 1963 *57* 284—300

FRANCIS B F Changes in the shape and size of the tracheobronchial tree following stimulat on the vago-sympathetic nerve *Arch Surg* 1929 *19* 1577—1583

KISS F Das Verhaltnis zwischen Vagus und Sympath cus *Anatomisch Histologische Unteru uber das Sympatische Nervensystem* 1932 *6* 129—150

[illegible]

1936 *15* 1723—1724

STERNSCHEIN E. Über Anastomosen zwischen Vagus und Sympathicus der Katze *Z Gesch.* 1922 *64* 441—444

THORNTON J W, Reactions of isolated bronchi *Quart J exp Physiol* 1937 *21*

WIDDICOMBE, J G Regulation of tracheobronchial smooth muscle *Physiol Rev* 19[illegible]

WYSS, O A M La mot li é de la paroi bronchique *Bronches* 1952 *2* 101—151

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The Adrenergic Nerves of Rat Salivary Glands after Excretory Duct Ligation

By

NILS ERIK ANDÉN, KARL AXEL NORBERG and LARS OLSON

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Abstract

ANDÉN, N E, K -A NORBERG and L OLSON *The adrenergic nerves of rat salivary glands after excretory duct ligation* Acta physiol scand 1966 *66* 501—506

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The disappearance of the sympathetic nerves and their neurohumoral transmitter noradrenaline (NA) from an organ after postganglionic sympathectomy has been of great value in physiological and pharmacological studies on adrenergic mechanisms Investigations of all types of neurohumoral transmission must, however, take into account also what happens postsynaptically Unfortunately the post synaptic structures cannot be selectively destroyed as easily as the presynaptic ones It has, however, been reported that the salivary gland cells atrophy after ligation of the excretory ducts (Junqueira 1951 Bhaskar, Bolden and Weinmann 1956 Standish and Shafer 1957) These findings have prompted the present study on the NA nerves in atrophied salivary glands using both a histochemical and biochemical approach

Material and Methods

Adult rats weighing about 250 g were used The results obtained were similar irrespective of differences in sex and strain (Sprague-Dawley hooded The rats were operated on under ether or pentobarbital sodium anesthesia By a skin incision in the midline of the neck the submaxillary and sublingual glands were exposed on either side The two glands occur in a common capsule a [illegible]

TABLE I Rat submaxillary gland, 14 days after ligation of the main excretory duct on one side The values are averages ± S E (n = 12)

	Weight in mg	Noradrenaline in $\mu g/g$	Noradrenaline in μg/gland
Gland on the side not ligated	224 ± 16 2	1 6 ± 0 15	0 35 ± 0 036
Gland on the side ligated	74 ± 5 2	3 7 ± 0 29	0 27 ± 0 033
Difference	P < 0 001	P < 0 001	P > 0 05

TABLE II Rat submaxillary plus sublingual glands 14 days after ligation of the main excretory ducts on one side The values are averages ± S E (n = 11)

	Weight in mg	Noradrenaline in $\mu g/g$	Noradrenaline in μg/gland
Glands on the side not ligated	303 ± 14 7	1 2 ± 0 11	0 36 ± 0 027
Glands on the side ligated	93 ± 7 0	2 6 ± 0 18	0 24 ± 0 014
Difference	P 0 001	P < 0 001	P < 0 001

sutured The animals were sacrificed by a blow on the head or bleeding out under light ether anesthesia, usually 7 or 14 days after the operation

Small pieces of the submaxillary and sublingual glands were freeze dried treated with formaldehyde gas embedded sectioned and examined under the fluorescence microscope (for references see Norberg and Hamberger 1964) For comparison some sections were stained with Azan or hematoxylin plus eosin (Romeis 1948) or astrablau (Bloom and Kelly 1960) and examined under a light microscope

For the biochemical determinations of NA and 5 hydroxytryptamine (5-HT) the submaxillary [illegible] by a [illegible] n the [illegible] HT [illegible] apfy [illegible] (Bertler 1961 Andén and Magnusson 1966

Results

The ligation of the main excretory ducts caused a reduction by about two thirds of the gland size (see Table I and II) The atrophy was of about the same magnitude for both the submaxillary and the sublingual gland The process seemed to be finished within 7 days

Light microscopy The *acini* of the atrophied submaxillary gland were markedly diminished in size The periglandular connective tissue had proliferated considerably and contained more inflammatory cells than usual The acinar cells had apparently intact nuclei but were very small due to a severely reduced cytoplasm The light zone with secretory granules near the lumen occurring in normal gland cells had vanished The cells, thus, appeared vital but inactive

The intralobular and interlobular *ducts* had also atrophied They could not be discriminated in the atrophied glands as the former had lost their striated appearance



Fig 1 Rat submaxillary gland normal Fine strongly fluorescent varicose adrenergic nerve terminals enclose the acini whereas one large interlobular duct and some small intralobular ducts are completely devoid of such an innervation There are also abundant adrenergic nerve terminals in the walls of some small blood vessels 180 ×

(mitochondria) in the basal portion of the cells The eosinophilic granules in the special cells of the intralobular ducts had also disappeared The duct epithelium had proliferated and sometimes it seemed to be multilayered The cell surface lining the lumen was bulging and the lumina were markedly dilated Like the acini the ducts were surrounded by an increased amount of connective tissue which contained many inflammatory cells and mast cells The latter could be specifically stained by astrablau

The sublingual gland displayed similar changes as the submaxillary gland — No major alterations occurred in either gland more than 7 days after the operation The glands on the side not ligated appeared intact at all intervals studied

Fluorescence microscopy The adrenergic innervation of normal rat submaxillary and sublingual glands as revealed by the fluorescence method for histochemical demonstration of monoamines has been described elsewhere (Norberg and Olson 1965) and is illustrated in Fig 1 A typical ground plexus of varicose adrenergic nerve terminals intimately surrounded acini of the serous submaxillary gland but not those of the mainly mucous sublingual gland All the ducts seemed to lack adrenergic innervation completely The blood vessels both in the submaxillary and sublingual glands had a prominent adrenergic innervation the nerves being located on the border between media and adventitia (*cf* Norberg and Hamberger 1964)

At no time could any detectable change be observed in the adrenergic innerva of the glands on the side not ligated In the atrophied glands the network of

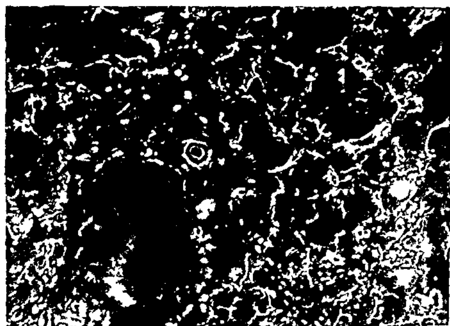


Fig 2 Rat subm ... ory duct The network of adrenergic nerve t ... (these are hardly ... near ... dilated excretory duct 100 ×

terminals was much denser (Fig 2) This finding was apparently due to the reduction of the volume of the acinar cells which could be distinguished by their faint background of fluorescence The terminals seemed to occur in bundles which usually seemed to contain more fibres than the normal 2—3 and therefore the individual terminals were often difficult to recognize Like in the normal glands the NA terminals enclosed the acini whereas the ducts were devoid of adrenergic innervation The adrenergic ground plexus of the blood vessels did not appreciably differ from the normal picture

In the interstitial connective tissue there were abundant mast cells with granules exhibiting yellow fluorescence due to their content of 5 HT The mast cells varied largely in size The absolute amount of mast cells in the atrophied gland appeared somewhat increased, as verified by astrablau staining (see above)

In addition to the above mentioned changes in specific amine fluorescence, autofluorescent granules were observed in increased amounts, scattered in the tissue of the atrophied glands

Biochemistry Normally the gland on the two sides had the same NA content and the same weight Nearly all the NA was in the submaxillary gland the sublingual gland containing at most only 0 2 µg g This is in agreement with the histochemical finding that the sublingual gland lacks an adrenergic innervation of the gland cells After excretory duct ligation the reduction of the glandular NA content was small compared to that of the glandular weight Therefore the concentration of NA was

increased more than twice in the submaxillary gland as well as in the submaxillary plus sublingual glands (Table I and II)

The concentration of 5 HT varied considerably (0 22—0 52 μg/g, n = 4) in the submaxillary plus sublingual glands on the side not ligated The level of 5 HT in the atrophied glands (0 64—1 58 μg/g) had increased so much that in all cases the content of 5 HT was larger than in the intact glands (2—5 times)

Discussion

Our light microscopical findings agree well with those previously reported (Junqueira 1951, Bhaskar, Bolden and Weinmann 1956, Standish and Shafer 1957) the atrophy was due to a conspicious reduction of the cytoplasm of the acinar cells whereas the ducts remained essentially intact and the interstitial connective tissue had proliferated It is unlikely that the atrophy was caused by a death of the acinar cells since the nuclei were only slightly changed and since the structure of the glands can recover almost completely after sectioning the duct ligature (Junqueira and Rabinovitch 1954) The present investigation shows that the salivary gland atrophy was not accompanied by a corresponding disappearance of the adrenergic nerves and their transmitter NA No doubt there was a certain reduction of the NA content in the atrophied glands but this phenomenon may be explained by technical errors, *e g* a pressure on the sympathetic nerves exercised by the ligature However it cannot be excluded that the length of the individual terminals has been somewhat reduced, concurrently with a much larger atrophy of the effector cells Anyhow, the loss of gland NA was always smaller than that of the gland weight — sometimes it was completely lacking — and therefore the NA level was increased between 2 and 3 times Histochemically the increase was even more striking

After excretory duct ligation there is thus a rather selective atrophy of the parenchymal cells in the salivary glands Such a preparation has been found to be valuable in studies on postsynaptic events of adrenergic transmission By a combined histochemical and biochemical approach we have found that the major part of the activity of the enzyme monoamine oxidase is present in the salivary gland cells and disappears at salivary gland atrophy (Almgren Anden Jonason Norberg and Olson 1965) It has also been observed that the uptake of injected tyramine and NA by the adrenergic nerves is very much reduced in atrophied rat salivary glands (Almgren Anden and Waldeck 1965) If the adrenergic nerves function normally in these glands this finding suggests that the amines are bound extraneuronally before entering the nerves

The increase of 5 HT containing mast cells in the atrophied salivary glands might be an inflammatory response as was the appearance of neutrophils and lymphocytes This view is supported by the finding that in a degenerating somatic nerve distal to a nerve section there is an accumulation of 5 HT containing mast cells in rats (Olsson 1965)

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References

Almgren O, N-E Anden J Jonason K-A Norberg and L Olson, Cellular localization of monoamine oxidase in rat salivary glands *Acta physiol scand* 1966 *67* In press

Almgren O N-E Anden and B Waldeck Extraneuronal binding as a possible factor of tyramine uptake by sympathetic nerves *Life Sci* 1965 *4* 121—126

Andén, N E and T Magnusson, To be publ

Bertler Å, Effect of reserpine on the storage of catechol amines in brain and other tissues *Acta physiol scand* 1961 *51* 75—83

Bertler Å A Carlsson and E Rosengren, A method for the fluorimetric determination of adrenaline and noradrenaline in tissues *Acta physiol scand* 1958 *44* 273—292

Bhaskar S N T E Bolden and J P Weinmann, Experimental obstructive adenitis in the mouse *J dent Res* 1956 *35* 852—862

Bloom G and J W Kelly The copper phtalocyanine dye 'Astrablue' and its staining properties especially the staining of mast cells *Histochemie* 1960 *2* 48—57

[illegible] on secret ng and resting

[illegible]

revealed by histochemical studies on the intraneuronal distribution of the transmitter *Acta physiol scand* 1964 *63* Suppl 238 1—42

Norberg K-A and L Olson Adrenergic innervation of the salivary glands in the rat *Z Zellforsch* 1965 *68* 183—189

Olsson Y Storage of monoamines in mast cells of normal and sectioned peripheral nerve *Z Zellforsch* 1965 *68* 255—265

Romeis B *Mikroskopische Technik* Munchen Leibniz Verlag 1948 pp 695

Standish S M and W G Shafer Serial histologic effects of rat submaxillary and sublingual salivary gland duct and blood vessel ligation *J dent Res* 1957 *36* 866—879

Acta physiol. scand. 1966, 66. 507—508

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Adrenergic Innervation of the Bronchial Muscle of the Cat

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It is well established that noradrenaline, and especially adrenaline, when administered to mammalian lungs through the vascular system cause bronchodilation (see Widdicombe 1963). Stimulation of the sympathetic nerves to the lung in the cat releases catecholamines into the venous effluent (Locket 1957) and generally causes bronchodilation (Dixon and Ransom 1912). The lack of specific histological methods has prevented a conclusive demonstration of the distribution of adrenergic nerves in the lung, although there have been strong indications for the existence of a double innervation of the bronchial muscle by both the sympathetic and the parasympathetic nervous systems (see Widdicombe 1963).

The bronchial innervation of the cat has now been studied by means of the histochemical method of Falck and Hillarp, which permits the specific visualization of the adrenergic innervation (Falck 1962, Falck *et al* 1962).

The bronchial muscle was found to have a general adrenergic innervation of high density. The adrenergic ground plexus (see Norberg and Hamberger 1964) seemed to be built up of single nerve terminals, closely approaching both the inner and outer surface of the muscle cells. This innervation extends as far as the respiratory bronchioles. The peribronchial ganglia were found to be composed of nerve cells lacking specific fluorescence and they can thus be concluded to be non-adrenergic (see Norberg and Hamberger 1964). No adrenergic nerve terminals, such as have been demonstrated in other parasympathetic ganglia (see Norberg and Sjöqvist 1965), were observed in these ganglia. Small arterioles accompanying the bronchioles had an adrenergic ground plexus of the usual type located on the border between the media and the adventitia. The innervation of the lung vessels will be described in full elsewhere.

The present histochemical findings in conjunction with physiological data indicate that activity in the sympathetic nerves to the lung in the cat causes bronchodilation by the release of catecholamines from adrenergic nerves constituting a rich innervation of the bronchial muscle.

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